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# Antimicrobial therapy of persistent *Anaplasma marginale* infections

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**Antimicrobial therapy of persistent *Anaplasma marginale* infections**

by

**Johann Francois Coetzee**

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:  
Michael D. Apley, Major Professor  
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Ames, Iowa

2005

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## DEDICATION

I wish to dedicate this thesis to my parents and close family who contributed immensely to my development as a person, veterinarian and a scientist. To my mom; I thank you for indulging my fascination with cows and for enthusiastically supporting my agricultural endeavors. It could not have been easy raising a farm kid in a city but you did a fabulous job. I will always have fond memories of driving down obscure country roads looking for toxic plants for my class collection. To my dad, I thank you for providing for my university education and for always encouraging me to pursue higher goals. I am proud that both of us concluded our doctoral studies in the same year. I have greatly valued your encouragement and wise counsel during these often challenging times. To my cousin Willie, the most talented stockman I have ever known. Thank you for humoring my first, often misguided, attempts at treating anaplasmosis. Not a day goes by where I don't use the many skills you taught me. Finally, I wish to acknowledge Him whose amazing creation has captivated and confounded me throughout my life and whose guidance has made all of this possible.

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## LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APHIS	Animal and Plant Health Inspection Agency
AUC	Area Under the plasma drug concentration versus time Curve
cELISA	Competitive Enzyme Linked Immunoabsorbent Assay
CF	Complement Fixation
CFIA	Canadian Food Inspection Agency
CMI	Cell Mediated Immune Response
COAC	Committee On Animal Care
CTC	Chorotetracycline
Dc	Doxycycline
DMSO	Dimethylsulphoxide
EB	Ethidium Bromide
ELISA	Enzyme Linked Immunoabsorbent Assay
ENRO	Enrofloxacin
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
HE	Hydroethidine
HGE	Human Granulocytic Ehrlichiosis
HGA	Human Granulocytic Anaplasmosis
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
HSD	Honest Significant Difference
IFA	Indirect Fluorescent Antibody Test
IM	Intramuscular administration
IMD	Imidocarb
IV	Intravenous administration
LIDIF	Livestock Infectious Diseases Isolation Facility
MANOVA	Multivariate Analysis of Variance
MASP	Microaerophilus Stationary Phase



MIC	Minimum Inhibitory Concentration
MRC	Minimum Rickettsiocidal Concentration
MSP	Major Surface Protein
nPCR	Semi Nested Polymerase Chain Reation
OIE	Office International des Epizooties
OK	Oklahoma isolate
OTC	Oxytetracycline
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PK/PD	Pharmacokinetic/Pharmacodynamic
PPE	Percent Parasitized Erythrocytes
RBC	Human Blood Cells
RCA	Rapid Card Agglutination test
RFCP	Restricted Feeder Cattle Program
ROC	Receiver Operating Characteristic curve
SEM	Standard Error of the Mean
StM	St. Maries isolate
VESA1	Variant Erythrocyte Surface Antigen
VGN	Virginia isolate

## ABSTRACT

*Anaplasma marginale* is one of the most prevalent tick-borne pathogens of cattle worldwide. Cattle that recover from acute anaplasmosis become carriers in which low or microscopically undetectable *A. marginale* rickettsemia persists. There are currently no antimicrobials approved for elimination of persistent infections. In this report we tested the efficacy of antimicrobial treatment regimens to clear persistent *A. marginale* infection from cattle. In the first study, administration of oxytetracycline at 30 mg/kg, intramuscularly (IM) once or twice 5 days apart was not effective for elimination of the persistent infections. Furthermore, the study demonstrated that the current recommended OIE treatment protocol of 5 injections of oxytetracycline administered at 22 mg/kg intravenously (IV) was also ineffective. In the second study we used flow cytometric analysis (FACS) to evaluate the effect of antimicrobials against *A. marginale* in short-term whole erythrocyte cultures. Enrofloxacin inhibited *A. marginale* in a concentration dependent manner, while higher concentrations of imidocarb were less effective in reducing the number of viable organisms. Oxytetracycline was found to be the least efficacious antimicrobial in this culture system. Cultures of erythrocytes infected with the Oklahoma isolate exposed to 4.0 µg/ml enrofloxacin and those of the Virginia and Oklahoma isolates exposed to 1.0 µg/ml appeared to be sterilized. Cultures exposed to 16 µg/ml oxytetracycline were not sterilized. As a result of these data we tested the efficacy of enrofloxacin (Baytril®100, Bayer Animal Health) against severe experimental *A. marginale* infections in splenectomized calves. These data indicate that enrofloxacin administered at 12.5 mg/kg twice, 48 h apart ameliorates, but does not clear, *A. marginale* infection in splenectomized calves. Finally we compared the efficacy of enrofloxacin, imidocarb and oxytetracycline against persistent *A. marginale* infections. The results suggest that apparent clearance of persistent infections occurred in one calf treated with imidocarb dipropionate at 5 mg/kg administered IM twice, 7 days apart, and one calf treated with oxytetracycline administered at 22 mg/kg q 24 h for 5 days. No calves treated with 5 mg/kg intravenous enrofloxacin for 5 days were cleared. Further studies are warranted to investigate whether dose regimens can be identified to reliably eliminate persistent *A. marginale* infections.

## CHAPTER 1. GENERAL INTRODUCTION

### Dissertation organization

This dissertation is organized into seven chapters. The remainder of this first chapter is a brief review of the relevance of research into the chemotherapy of persistent bovine anaplasmosis infections and concludes with a statement of the guiding hypotheses studied in this report. Chapter 2 is a literature review designed for publication. This is written as a critical review of methods for chemosterilization of persistent intra-erythrocytic hemoparasitic infections in cattle. Chapter 3 is the first research paper from this doctoral project. This study tested the efficacy of three oxytetracycline treatment regimens to clear *Anaplasma marginale* from cattle that were persistently infected. Chapter 4 is the second research paper in which we used flow cytometric analysis (FACS) to evaluate the effect of antimicrobials against *A. marginale* in short-term whole erythrocyte cultures. The third research paper is presented in chapter 5, which contains the results of a study conducted to test the efficacy of enrofloxacin (Baytril®100, Bayer Animal Health) against severe experimental *A. marginale* infections in splenectomized calves. In the final research paper presented in chapter 6, we compared the efficacy of enrofloxacin, imidocarb and oxytetracycline against persistent *A. marginale* infections. Chapter 7 presents general conclusions drawn from the research described in this dissertation. Also included is a brief description of recommendations for continued research.

The coauthors of the submitted manuscripts contributed in the following ways: Michael D. Apley was the major professor and was involved in all aspects of the study design, data collection and analysis. Katherine M. Kocan from Oklahoma State University and her technicians conducted the examination of stained blood smears for determination of parasitemia. She also consulted on study design and data collection and provided isolates for experimental infection of study animals. Fred R. Rurangirwa from Washington State University conducted the polymerase chain reactions (PCR) in the first and fourth study. Joyce Van Donkersgoed from the Alberta Cattle Feeders Association was the study monitor and represented the sponsor. Doug Jones was an integral collaborator in the development of

the flow cytometry method, the performance of flow cytometry and the analysis of FACS data.

### **Relevance of research into the chemotherapy of persistent *A. marginale* infections**

Anaplasmosis, caused by the rickettsial hemoparasite, *A. marginale*, is one of the most prevalent tick-transmitted diseases of cattle worldwide (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2003). The cost of a clinical case of anaplasmosis in the United States of America has been conservatively estimated to be over \$400 per animal (Goodger et al., 1979; Alderink et al., 1982). Kocan et al. (2003) in a recent review article estimated the cost of anaplasmosis in the USA to be over \$300 million per year. Anaplasmosis is currently classified in List B of the Office International des Epizooties (OIE) Terrestrial Animal Health Code due to its socio-economic importance and significance in terms of restrictions in the international trade of animals and animal products (OIE, 2003).

*A. marginale* is transmitted via biological and mechanical vectors such as ticks and biting flies, and also by contaminated fomites such as needles and surgical instruments (Dikmans, 1950). The acute phase of the disease can cause severe anemia due to extra-vascular hemolysis, abortions, weight loss and death (Potgieter and Stoltz, 1994). Cattle that recover from acute anaplasmosis remain persistently infected with *A. marginale*. Persistent infection is characterized by sequential rickettsemic cycles ranging from  $10^2$ - $10^7$  parasitized erythrocytes that occur at approximately 5-week intervals (Kieser et al., 1990). During these cycles, infected erythrocytes are not always detectable in stained blood smears, but ticks are able to acquire *A. marginale* infection from carrier cattle (Eriks et al., 1989). Thus, carrier animals serve as reservoirs of infection for mechanical transmission and infection of ticks which are biological vectors (Reeves and Swift, 1977; Eriks et al., 1989; Futse et al., 2003).

The ability of *A. marginale* to persist in a fully immunocompetent host suggests that persistence involves a mechanism of escape from the immune response (Barbet et al., 2000). The identification of repeated cycles of rickettsemia, each composed of a progressive, logarithmic increase in organisms, followed by a precipitous decrease, led to the hypothesis

that persistence reflects the sequential emergence and subsequent immune control of antigenic variants (Palmer et al., 2000).

Distinct outer membrane proteins, termed major surface proteins (MSPs) have been identified on *A. marginale* organisms derived from bovine erythrocytes (Barbet, 1995). These proteins are targets for the host immune response and can also be used in diagnostic assays. MSP1 has been reported to be an adhesin for bovine erythrocytes and tick cells and affects the ability of the organism to be transmitted by ticks (de la Fuente et al., 2002). This protein has been shown to confer partial protection in immunized cattle. Both MSP2 and MSP3 are encoded by large polymorphic multigene families. The MSP2 sequence specifically varies during cyclic rickettsemia. It has been demonstrated that unique MSP2 structural variants arise in each cycle of persistent rickettsemia and that a specific immune response to MSP2 is associated with organism clearance (French et al., 1998). Variance in MSP3 has been shown between geographic locations (Kocan et al., 2000). Little is known about MSP4, but MSP5 has been shown to be conserved on all isolates of *A. marginale* and has proven to be a good diagnostic antigen for use in a newly developed competitive ELISA test (Torioni De Echaide et al., 1998).

Chlortetracycline (CTC) and oxytetracycline (OTC) are the only approved compounds used clinically for treatment of anaplasmosis in the United States. Oxytetracycline is a tetracycline derivative obtained from *Streptomyces rimosus*. Tetracyclines are bacteriostatic antibiotics that inhibit protein synthesis by reversibly binding to 30S ribosomal subunits of susceptible organisms (Plumb, 2002). However, *A. marginale* infections were not cleared from cattle using recommended therapeutic doses of these compounds (Kuttler and Simpson, 1978; Stewart et al., 1979). Previous studies, in which successful clearance of persistent *A. marginale* infections was reported, administered oxytetracycline intravenously to cattle at 11–22 mg/kg for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Intramuscular oxytetracycline administered at 20 mg/kg on two, three or four occasions at intervals ranging from 3–7 days was also reported to be effective at eliminating carrier infections (Roby et al., 1978; Kuttler, 1980; Magonigle and Newby, 1982; Kuttler, 1983; Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem et al., 1988).

The existence of persistent *A. marginale* infections in cattle despite treatment restricts the movement of animals from areas where the disease is prevalent to areas where the disease is not regarded as endemic such as Canada. Prior to the publication of the paper presented in chapter 3, only limited movement of feeder cattle from selected northern states was permitted under the Restricted Feeder Cattle Program (RFCP) (Canadian Food Inspection Agency, 2002). The RFCP facilitates the export of feeder cattle from the U.S.A to Canada between 1 October and 31 March (the non-vector season) provided these animals receive oral tetracycline for 120 days or 2 injections of a long acting oxytetracycline formulation at a dose rate of 20 mg/kg on arrival and 5–7 days later. As a consequence of this, the Canadian beef industry was looking for alternative treatments, for example a single injection of long-acting oxytetracycline, to reduce the current cost of repeat treatments and the risk of antimicrobial resistance development from long-term use of feed medications. If a single treatment of long-acting oxytetracycline is effective or none of the treatments are effective, then the RFCP may need to be modified accordingly.

### **Guiding hypotheses of this dissertation**

The hypothesis of the first paper presented in this dissertation was that either a single injection of a new long-acting OTC formulation (300 mg/ml OTC) administered at 30 mg/kg bodyweight or 2 injections administered 5 days apart IM would be as efficacious as the current OIE recommended treatment protocol which requires the administration of 22 mg/kg OTC IV for 5 days. In this paper data are presented that support the rejection of this hypothesis and also demonstrate that the OIE recommended treatment protocol was ineffective.

As a result of these findings we hypothesized that the failure to sterilize persistent *A. marginale* infections could be attributed to one or more of the following factors: (1) drug concentrations at the site of infection were inadequate, (2) drug concentration was maintained for an insufficient duration to be effective, (3) the organism was not susceptible to the antimicrobial, (4) the pharmacokinetic parameters of the drug were inadequate, and (5)

local factors in the environment where the organism was located were not conducive for optimal drug activity (Bidgood and Papich, 2003).

In the second experiment we hypothesized that an *in vitro* whole blood culture system for *A. marginale* previously described by Kessler et al. (1979) could be used in conjunction with a fluorescent activated cell sorting (FACS) method described by Wyatt et al. (1991) to evaluate antimicrobials against *A. marginale*. Once established, this technique could also be used to test the hypothesis that anaplasmosis isolates from different geographic locations differ in their susceptibility to antimicrobials. This study demonstrated that enrofloxacin (ENRO) inhibited *A. marginale* in a concentration dependent manner, while higher concentrations of imidocarb (IMD) were less effective in reducing the number of viable organisms. Oxytetracycline (OTC) was found to be the least efficacious antimicrobial in this culture system. Differences between isolates were evident at some dilutions. Cultures of erythrocytes infected with the Oklahoma isolate exposed to 4.0 µg/ml enrofloxacin and those of the Virginia and Oklahoma isolates exposed to 1.0 µg/ml imidocarb appeared to be sterilized. This study appears to be the first *in vitro* study demonstrating the anti-*A. marginale* microbial effect of enrofloxacin.

Based on the results of the *in vitro* trial we hypothesized that enrofloxacin could potentially be used to sterilize persistent *A. marginale* infections. Accordingly we designed the study reported in chapter 5 to test the efficacy of enrofloxacin (Baytril®100, Bayer Animal Health) against severe experimental *A. marginale* infections in splenectomized calves. The results of this study indicated that enrofloxacin administered at 12.5 mg/kg twice, 48 h apart ameliorates, but does not eliminate, *A. marginale* infection in splenectomized calves.

Finally we compared the efficacy of enrofloxacin, imidocarb and oxytetracycline against persistent *A. marginale* infections established experimentally using isolates from Oklahoma (OK), Virginia (VGN) or Idaho (St Maries; StM). One calf infected with the OK isolate treated with imidocarb dipropionate at 5 mg/kg administered IM twice, 7 days apart, and one calf infected with the VGN isolate treated with oxytetracycline administered at 22 mg/kg q 24 h for 5 days failed to develop parasitemia following splenectomy. Subinoculation of blood pooled from these calves failed to infect a splenectomized calf. The OTC treated

calf and the subinoculated splenectomized calf was negative for the parasite on PCR and nPCR, but the IMD treated calf was positive. These data indicate that two of the regimens described were efficacious against persistent *A. marginale* infections in only two animals, one infected with an OK isolate and one infected with a VGN isolate. No calves treated with 5 mg/kg intravenous enrofloxacin for 5 days were cleared of infection.

Taken together, these studies suggest that reliable clearance of persistent *A. marginale* infections could not be achieved with oxytetracycline, imidocarb dipropionate or enrofloxacin at the dose, route, frequency and duration of administration tested. We also showed that flow cytometry proved to be useful for screening antimicrobial activity of drugs against *A. marginale* infected bovine erythrocytes. Based on the data obtained from these analyses we were able to make predictions about the minimum inhibitory concentration of antimicrobials required to be effective against anaplasmosis. We also demonstrated *in vitro* susceptibility differences between a Virginia and Oklahoma isolate especially in the imidocarb treated cultures. Enrofloxacin was also identified as a compound that could potentially be used against *A. marginale*. Further research is required to investigate why current chemosterilization protocols against *A. marginale* provide inconsistent results. This may require further elucidation of the relationship between pharmacotherapy and the immune system, and the determinants of isolate susceptibility differences.

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## CHAPTER 2. A CRITICAL REVIEW OF METHODS FOR CHEMOSTERILIZATION OF PERSISTENT INTRA-ERYTHROCYTIC HEMOPARASITIC INFECTIONS IN CATTLE

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### Abstract

Babesiosis and anaplasmosis are tick transmitted diseases caused by obligate intraerythrocytic hemoparasites. It has been estimated that at least 500 million cattle are exposed to babesiosis and anaplasmosis worldwide. Following acute infection, both diseases establish persistent infections that confer lifelong immunity to the host. The existence of a subclinical carrier state in cattle poses a risk when animals are moved from endemic to non-endemic regions. For this reason these diseases are currently classified in List B of the Office International des Epizooties (OIE) Terrestrial Animal Health Code due to their socio-economic importance and significance in terms of restrictions in the international trade of animals and animal products. Current OIE recommendations propose that chemosterilization of babesiosis infections can occur with an effective drug such as imidocarb as a single dose injection at 2 mg/kg. Persistent *A. marginale* infections are purported to be sterilized with an effective drug such as oxytetracycline for 5 consecutive days at a dose of 22 mg/kg prior to export. However, recent studies have failed to eliminate anaplasmosis infections in experimentally infected carrier animals using this treatment regime. The purpose of this review paper is to examine the antimicrobials currently available for chemosterilization of persistent *Babesia* and *Anaplasma* infections in cattle and the publications used to support these recommendations. The next section will focus on possible reasons why the recommended antimicrobial therapies are unsuccessful. Finally, future developments and potential areas for continued research into chemosterilization protocols are discussed.

## Introduction

The tick-borne hemoparasitic diseases of greatest global economic importance in cattle are babesiosis, anaplasmosis, theileriosis and cowdriosis (Uilenberg, 1995). Of these, only anaplasmosis, caused by *Anaplasma marginale* and babesiosis, caused by *Babesia bigemina* and *Babesia bovis*, are intimately associated with erythrocytes and will therefore be the main focus of this review. Both diseases are currently classified in List B of the Office International des Epizooties (OIE) Terrestrial Animal Health Code due to their socio-economic importance and significance in terms of restrictions in the international trade of animals and animal products (OIE, 2004a, 2004b).

Following acute disease, *A. marginale* establishes lifelong persistent infections. These infections are characterized by sequential rickettsemic cycles ranging from  $10^2$ – $10^7$  parasitized erythrocytes that occur at approximately 5-week intervals (Kieser et al., 1990). Each cycle arises due to antigenic variation of the immunodominant outer membrane protein MSP2, which occurs by combinatorial gene conversion (Brayton et al., 2002).

Similarly, cattle recovering from babesiosis retain a latent infection lasting six months to several years (De Vos et al., 2004). Unlike anaplasmosis, recrudescence of parasitemia occurs at irregular intervals (Mahoney, 1977). At least 5 mechanisms have been identified which might contribute to evasion of host immunity by *Babesia* organisms. These include: (1) antigenic variation of a variant erythrocyte surface antigen (VESA1), (2) cytoadhesion/sequestration, (3) binding host IgM to the surface of infected erythrocytes, hiding the parasite from immune recognition, (4) monoallelic expression of different members of multigene families, and (5) induction of transient immunosuppression (Allred, 2003).

Maintenance of persistent infections confers lifelong immunity to the host. The carrier state is also important in endemic areas as it contributes to endemic stability of the disease. Under these circumstances young animals are exposed to infection before passive protection from maternal antibody wanes. Consequently, serious disease outbreaks do not occur even though the causal organism is widely distributed in the population (Sergent, 1965; Mahoney, 1977).

The existence of persistent infections in cattle, in many cases despite treatment with prescribed antimicrobial protocols, poses a risk when animals are moved from endemic to non-endemic regions. In light of this, Article 2.3.8.2 of the OIE Terrestrial Animal Health Code states that when importing cattle from countries considered infected with bovine babesiosis, Veterinary Administrations of free countries should require the presentation of an international veterinary certificate attesting that the animals (1) showed no clinical sign of bovine babesiosis on the day of shipment; and (2) were, since birth, resident in a zone known to be free of bovine babesiosis for the previous 2 years; OR (1) showed no clinical sign of bovine babesiosis on the day of shipment; and (2) were subjected to a diagnostic test for bovine babesiosis with negative results during 30 days prior to shipment; and (3) were treated with an effective drug such as imidocarb as a single dose injection at 2 mg/kg or amicarbalide at 10 mg/kg (under study) (OIE, 2004a).

Recent studies that independently verify these recommendations are lacking in the published literature. Amicarbalide is no longer commercially available and will therefore not be examined in this review (Cooper and Watson, 1989). Essentially the recommendations involving imidocarb are based on work conducted by Callow and McGregor (1970), Callow et al. (1974a) and Lewis et al. (1981). Since these studies were conducted, it has been recognized that the larger babesias (*B. bigemina*) appear to be more sensitive to imidocarb than the smaller organisms like *B. bovis* (Kuttler and Johnson, 1986). Furthermore, newer molecular technologies such as PCR and DNA hybridization have been developed that would assist in identifying persistent infections.

In addition to the recommendations stated under Article 2.3.8.2 for anaplasmosis, Article 2.3.7.2 of the terrestrial animal health code states that animals can be treated with an effective drug such as oxytetracycline for 5 consecutive days at a dose of 22 mg/kg (under study) prior to export (OIE, 2004a). In previous studies where successful clearance of persistent *A. marginale* infections was reported, oxytetracycline was administered intravenously to cattle at 11–22 mg/kg for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Intramuscular oxytetracycline administered at 20 mg/kg on 2, 3 or 4 occasions at intervals ranging from 3–7 days was also reported to be effective at eliminating carrier infections (Roby et al., 1978; Kuttler, 1980; Magonigle and Newby, 1982; Kuttler, 1983;

Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem et al., 1988). A recent study conducted by our research group demonstrated that the current recommended OIE treatment protocol of 5 daily injections of oxytetracycline administered intravenously at 22 mg/kg did not eliminate persistent Oklahoma isolate infections (Coetzee et al., 2005). It therefore seems appropriate to re-evaluate many of the prescribed treatment regimens for clearing persistent infections especially in light of many of the recent advances in molecular technologies.

This review paper is divided into 4 sections. Section 1 will review the global economic significance of anaplasmosis and babesiosis. Section 2 will review the antimicrobials recommended for chemosterilization and the publications used to support these recommendations. Section 3 will focus on possible reasons why the recommended antimicrobial therapies are unsuccessful. Finally, section 4 will speculate on future developments and potential areas for continued research into chemosterilization protocols.

## **1. Economic significance of anaplasmosis and babesiosis**

Anaplasmosis, caused by the rickettsial hemoparasite *Anaplasma marginale*, is one of the most prevalent tick-transmitted diseases of cattle worldwide (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2003). Losses due to anaplasmosis arise from anemia, icterus and fever associated with clinical infections and occasional abortions. The disease has been reported in Africa, North and South America, Russia, European countries bordering the Mediterranean and the Middle and Far East (Kocan et al., 2000).

The incidence of clinical anaplasmosis has been estimated to be 1.85% in beef cattle in California (Goodger et al., 1979), 0.276% in Texas (Alderink and Dietrich, 1982) and up to 31% in Louisiana (Morley and Hugh-Jones, 1989). It is estimated that the introduction of anaplasmosis into a previously naïve herd can result in a 3.6% reduction in calf crop, a 30% increase in cull rate and a 30% mortality rate in clinically infected adult cattle (Alderink and Dietrich, 1982). The cost of a clinical case of anaplasmosis in the United States has been conservatively estimated to be over \$400 US per animal (Goodger et al., 1979; Alderink et al., 1982). Kocan et al. (2003) in a recent review article estimated the cost of anaplasmosis in the U.S. to be over \$300 million per year.

In the North American context, the existence of persistent *A. marginale* infections in cattle, despite treatment, restricts the movement of breeding animals from the U. S. to Canada where the disease is not regarded as endemic. The losses associated with *A. marginale* becoming established in Canada are estimated between \$12.1 and \$36.0 million CAD. Meanwhile, the estimated cost to the Canadian Food Inspection Agency (CFIA) to prevent the spread of the disease is \$3 million CAD should anaplasmosis be introduced (CFIA, 2003).

In March, 2004, the Canadian government removed anaplasmosis and bluetongue testing and treatment requirements from feeder cattle imported from most U.S. states (CFIA, 2003). Although these new provisions facilitate year-round movement of feeder cattle from 39 U.S. states to Canada, there are control provisions for the movement of imported feeders between approved feedlots and for Canadian cattle to leave importing feedlots to re-enter Canada's breeding herd. Imported animals are eligible to leave the feedlot only for immediate slaughter, movement to another feedlot that is approved to import restricted feeder cattle or to be re-exported to the U.S. There are requirements for comprehensive management programs for animals in the feedlot, enhanced insect vector control programs and periodic inspection of animals within importing feedlots.

*Babesia bovis* (European Redwater) and *Babesia bigemina* (African Redwater) are responsible for the most significant economic losses associated with babesiosis. In addition to fever, hemoglobinuria and anemia associated with intravascular hemolysis, *B. bovis* is responsible for neurological signs associated with sequestration of erythrocytes in the cerebral vasculature (De Vos et al., 2004). Abortions in pyrexia animals have also been recorded.

Babesiosis persists in tropical and semitropical regions between the 32nd parallel in the south to the 40th parallel north of the equator. Babesiosis or "Texas fever" was eradicated from the U.S. in 1943, following implementation of intensive acaricide dipping to eliminate *Boophilus annulatus* from cattle in accordance with the Animal Industry Act of 1884. In 1976 the benefit/cost ratio for the Texas program was estimated to be 172 to 1. The direct cost of maintaining this barrier was estimated around \$5,276,000 in 2001 (APHIS, 2005).



However, it is estimated that the first year cost of controlling vector ticks alone should they be introduced into the U.S. is over \$1.3 billion (Washington State University, 2005).

De Vos (1992) estimated that at least 500 million cattle are exposed to babesiosis and anaplasmosis worldwide. Based on reports from Argentina, Mexico and Australia, it has been estimated that annual loss of five dollars (US) per head is due to ticks and tick-borne diseases (Buening, AFIP website). There are approximately 175 million cattle residing in tick-infested areas of Central and South America; thus the cattle industry of Latin America experiences a total loss of \$875 million (US) per annum. Worldwide the cost is estimated to be around \$2.5 billion (US). Additional economic costs are related to quarantine and control measures such as restriction of cattle movement from endemic to non-endemic zones.

## **2. Antimicrobials currently available for chemosterilization of persistent anaplasmosis and babesiosis infections**

### ***Introduction***

Prior to the development of the tetracycline antimicrobials and the diamidine derivatives, a variety of chemotherapeutic agents, including arsenicals, antimalarials, antimony derivatives and dyes, were used to treat acute anaplasmosis and babesiosis in cattle. These compounds had little if any chemotherapeutic effect, with the exception of trypan blue which was the first specific drug used to successfully treat babesiosis (Kuttler, 1988; Potgieter and Stoltz, 1994). However, trypan blue discolors flesh and body secretions therefore giving it limited usefulness in food animals. This compound also fails to sterilize infections.

Chlortetracycline (CTC) and oxytetracycline (OTC) are the only compounds approved for use against acute anaplasmosis in the U.S. However, *A. marginale* infections are not sterilized at the usual recommended therapeutic doses of these compounds (Kuttler and Simpson, 1978; Stewart et al., 1979). There are currently no antimicrobials labeled for the elimination of persistent *Anaplasma* infections in carrier animals. Previous studies have reported chemosterilization of *A. marginale* infections using tetracycline and oxytetracycline IV or IM, chlortetracycline IV or orally, and imidocarb as a single therapy or in combination with  $\alpha$ -ethoxyethylglyoxal dithiosemicarbazone (Gloxazone) (reviewed by Kuttler, 1980).

However, gloxazone was never made commercially available due to concerns relating to the toxicity of this compound in lactating cows (Kuttler, 1971).

Imidocarb dipropionate (Imizol, Schering Plough) and diminazene aceturate (Berenil, Hoechst Ltd.) are the most common agents used in the chemotherapy of bovine babesiosis. Previously, quinuronium sulfate and amicarbilide isoethionate were available as therapeutic options, however, these have been withdrawn because of manufacturing safety issues (Cooper and Watson, 1989; Zintl et al., 2003). Imidocarb and diminazene aceturate are also not available for use in food animals in the U. S. due to prolonged tissue levels and potential carcinogenic effects (EMEA, 2001; Kuttler, 1988).

### *Tetracycline antimicrobials*

#### *Pharmacology*

The tetracycline antimicrobials have been extensively reviewed by Scholar and Pratt (2000) and Chopra and Roberts (2001). What follows is a brief summary of the pertinent information contained within these reviews.

Chlortetracycline (CTC) and oxytetracycline (OTC), both discovered in the late 1940s, were the first members of the tetracycline group to be described. Tetracycline molecules comprise a fused tetracyclic nucleus to which a variety of functional groups are attached. Barragry (1994) and Scholar and Pratt (2000) reviewed the mechanism of uptake of tetracyclines by bacterial microorganisms. The tetracyclines are accumulated in both Gram-positive and Gram-negative bacteria in an energy dependant manner. Two types of uptake systems are involved in tetracycline accumulation. There is an initial, rapid uptake that is driven by a proton-motive force caused by the difference in the pH between the environment and the bacterial cytoplasm (Nikaido and Thanassi, 1993). This mechanism appears to reach equilibrium with the tetracycline in the medium in about 5 min.

The second uptake system is a slower energy-dependant uptake that accumulates tetracycline over a period of hours. In Gram-negative bacteria, the tetracyclines pass through the outer membrane through the OmpF and OmpC porin channels as positively charged cations probably in complex with magnesium (Chopra et al., 1992). In Gram-positive bacteria it is assumed to be the electroneutral, lipophilic form that crosses the membrane.

Within the cytoplasm, tetracycline molecules are likely to become chelated since the internal pH and divalent metal ion concentrations are higher than those outside the cell (Schnappinger and Hillen, 1996). The mechanism of entry of tetracyclines into rickettsia has not been elucidated.

Tetracyclines are bacteriostatic agents with broad spectrum antimicrobial activity. Tetracycline activity has been demonstrated against Gram-positive and Gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae and protozoan parasites. They are also used prophylactically for the prevention of malaria caused by mefloquine-resistant *Plasmodium falciparum* (Chopra and Roberts, 2001). There has been widespread emergence of efflux- and ribosome-based resistance to chlortetracycline and oxytetracycline in veterinary and human medicine, which has been hypothesized to be due to the widespread and sometimes indiscriminate use of these compounds (Acar, 1997; Chopra et al., 1992).

The tetracyclines bind to ribosomes and mRNA. However, the inhibition of protein synthesis is mediated principally through reversible binding with the 30S ribosomal subunit. Six tetracycline-binding sites, with no common structural trait and which demonstrate different binding affinities, have been identified on the 30S subunit (as reviewed by Auerbach et al., 2002). In bacteria, the primary binding site lies in a clamp-like pocket formed by the head, at the A site of the mRNA-ribosome complex. The tetracycline at this site interacts with the sugar-phosphate backbone of H34 through a magnesium ion. Reversible binding of tetracycline to the A-site blocks the attachment of aminoacyl-tRNA. The reversible nature of this association provides an explanation for the bacteriostatic effects of the drug (Chopra et al., 1992). The significance of the remaining five binding sites is not known, but it has been hypothesized that these could act synergistically to contribute to the bacteriostatic effect of the drug. The four proteins that interact with tetracycline at these alternate sites are primary rRNA binding proteins. Disruptions of these may therefore disturb the assembly steps of the 30S subunit.

Tetracyclines, as a class, are widely distributed in the body with a volume of distribution of 0.8 L/kg reported in cattle. The amount of plasma protein binding for oxytetracycline is about 10–40%. Pharmacokinetic studies in cattle have found that the

maximum plasma concentration ( $C_{\max}$ ) following IM administration of a long acting formulation containing 200 mg/ml oxytetracycline at a dose rate of 20 mg/kg bodyweight was 8.56  $\mu\text{g/ml}$  achieved at 2.53 h after injection. The AUC was 236  $\mu\text{g/ml}\cdot\text{h}$  and the elimination half time ( $T_{1/2}$ ) was 16.5 h. The  $C_{\max}$  following IM administration of a long acting formulation containing 300mg/ml oxytetracycline at a dose rate of 30 mg/kg bodyweight was 10.72  $\mu\text{g/ml}$  achieved at 4.64 h after injection. The AUC was 384  $\mu\text{g/ml}\cdot\text{h}$  and the elimination half time ( $T_{1/2}$ ) was 20.8 h (Clark and Dowling, 2003). Judging this data set probably overestimates both curves.

In a study reported by the manufacturer, American Cyanamid, aureomycin (CTC) was fed orally to 6 Angus heifers (ave. 425 lbs. bodyweight) at a dose rate of 5 mg/lb (11 mg/kg) in 4 lb of concentrate for 4 days (American Cyanamide Company, 1991). A peak plasma drug concentration of approximately 0.2  $\mu\text{g/ml}$  was achieved approximately 12 h after feeding. Studies to independently confirm these data in the peer-reviewed literature are lacking.

Nouws et al. (1983) found that OTC exhibits age-dependent pharmacokinetics in ruminants. Following intravenous administration of OTC at 7.5 mg/kg bodyweight to 3-week-old calves, the AUC was 3569 mg/min $\cdot$ L and the  $T_{1/2}$  was 13.5 h. The volume of distribution ( $V_{d(\text{area})}$ ) in calves was  $2.48 \pm 0.19$  L/kg. In adult dairy cows, at drying off, administered OTC intravenously at 7.94 mg/kg bodyweight, the AUC was 8774 mg/min $\cdot$ L and the  $T_{1/2}$  was 10.3 h. The volume of distribution ( $V_{d(\text{area})}$ ) in calves was  $0.8 \pm 0.11$  L/kg. These authors surmised that the difference in AUC was related to decreases in  $V_{d(\text{area})}$  and plasma clearance associated with decreases in extracellular and total body water. They concluded that the recommended dose levels of OTC in young calves should be twice those employed in cows for obtaining similar plasma OTC concentration-time profiles. Differences in volume of distribution were also reported by Ames et al. (1983) who compared the pharmacokinetics of OTC in sick and healthy calves.

Cunha and Mattoes (2002) reviewed the pharmacodynamics of tetracyclines that were described as time-dependant. In other words, the time that plasma drug concentrations remain above the minimum inhibitory concentration (MIC) of the pathogen ( $T > \text{MIC}$ ) is the best predictor of antimicrobial activity. These authors state that if the area under the plasma

concentration versus time curve (AUC) approaches 2–4 times the MIC value of the organism, the bacteriostatic effect is maximized. Generally for these agents the  $T > MIC$  should be at least 50% of the dosing interval for immunocompetent patients, whereas immunocompromised patients may require  $T > MIC$  for 100% of dosing interval. The specific pharmacodynamics of OTC against rickettsial organisms has not been described in the published literature.

### ***Chemosterilization of persistent *A. marginale* infections with tetracyclines***

#### ***Tetracyclines administered by injection***

Foote and Wulf (1952) were the first to report successful chemosterilization of *A. marginale* infections using aureomycin (Chlortetracycline). This study treated 3 animals with a 2% solution of aureomycin. The first cow was treated during the acute phase of the disease when the percent parasitized erythrocytes (PPE) was 65%. An initial dose of 10 mg/lb (22 mg/kg) was administered followed by a dose of 11 mg/kg and a third dose of 22 mg/kg administered at 24-hour intervals. Two days later the cow received 11 mg/kg, and 8 h later a further 5.5 mg/kg aureomycin. Blood from this cow was inoculated into two susceptible splenectomized calves, 30 days apart. Neither calf developed anaplasmosis.

The second cow in this experiment had been classified as a carrier for 4 years. This cow received an initial dose of 13.6 mg/lb (30 mg/kg) followed by a second injection of 15 mg/kg administered 12 h later. On the second day, 10 h after the second injection, this cow received 9.09 mg/lb (20 mg/kg) followed by another 6.81 mg/lb (15 mg/kg) administered 12 h later. A final injection of 15 mg/kg was administered 12 h after the 4<sup>th</sup> injection. The third cow in this experiment had been a carrier for 4 months and was treated initially with 17.86 mg/lb (40 mg/kg) followed by an injection of 7.14 mg/lb (16 mg/kg) 12 h later. On the second day this cow received 10.71 mg/lb (24 mg/kg) followed by another injection of 16 mg/kg 12 h later. This was repeated for the last time 12 h after the 4<sup>th</sup> injection. Six days after treatment 40 ml of blood from each animal was subinoculated into splenectomized calves. Neither calf developed clinical anaplasmosis.

There are a number of factors that confound this first report. Essentially this is a case report as animals were not randomly assigned to treatment groups. At the outset, the route of

administration was not specified although presumably this was IV. Furthermore, there was inconsistency in the dose regimens that were used and there was no control group. In addition there were only three animals tested, only two of which were carriers. Based on the pharmacokinetics of tetracycline it is likely that animals may still have had circulating drug levels at the time when blood was inoculated into splenectomized calves 6 days after treatment. Combined, these factors serve to limit any inferences that can be made from these results. These deficiencies became more apparent when Splitter and Miller (1953) later reported that subinoculation of blood from the cows treated in this experiment proved infectious at 67 days after treatment.

Splitter and Miller reported the apparent eradication of the anaplasmosis carrier state in a study conducted in 1953. Ten animals ranging from 80–1,100 lbs were infected with carrier blood. These were confirmed infectious by subinoculation of blood into splenectomized calves at the time of treatment, which occurred between 33 and 180 days after infection. Complement fixation tests were also run to confirm seropositivity. Two calves received terramycin (oxytetracycline) at 5 mg/lb/day (11 mg/kg) administered in divided doses IV for 14 days. Blood from both animals was not infectious when inoculated into splenectomized calves after 240 days post-treatment. One calf received terramycin (oxytetracycline) at 1 mg/lb/day (2.2 mg/kg/day) IV for 16 days. This treatment failed. Three calves received terramycin (oxytetracycline) by IM injection at 11 mg/kg/day for 14 days. One of these received divided doses. Blood from these animals was not infectious when inoculated into splenectomized calves after 60 days post-treatment. One calf received terramycin (oxytetracycline) at 11 mg/kg/day IM for 12 days. This animal was also negative for anaplasmosis at 60 days. Two calves received 15 mg/lb/day (33 mg/kg) aureomycin IV for 16 days and one calf received 2.5 mg/lb/day (5.5 mg/kg) for 20 days. The two calves treated at the higher dose failed to infect a splenectomized calf at 16 days while the animal treated at the lower dose was infectious.

This case report is also confounded by inconsistency in the dose regimens that were applied. In addition to this there were very few replicates tested, which makes comparisons difficult. However, in contrast with the study conducted by Foote and Wulf (1952), animals were monitored for an extended period of time after treatment and the results therefore

appear to be credible. One animal appears to have been cleared of infection, as it proved susceptible to re-infection 1 year after treatment.

Pearson et al. (1957) studied the use of tetracycline against persistent *A. marginale* infections in 30 cattle naturally exposed to infection in Oklahoma. Carrier status was determined by a history of clinical anaplasmosis, subinoculation or positive CF test. Cattle were divided into three treatment groups comprising 10 animals per group. Within each group five animals was treated by IV injection and five animals received intramuscular injections. Group A received tetracycline at 5 mg/lb (11 mg/kg) once a day for 5 days. Group B received 11 mg/kg once daily for 5 days, were rested for 5 days, and then received an additional 5 days of tetracycline at the same dose rate. Group C received 11 mg/kg tetracycline for 10 consecutive days. Blood was subinoculated into splenectomized calves at intervals ranging from 52–237 days after treatment. Intramuscular treatment was successful in all lots. Of those cattle treated intravenously, chemosterilization was successful in two out of five cattle in group A, 4 out of 5 cattle in group B and all the cattle in group C. Based on these data the administration of tetracycline at 11 mg/kg once daily for 10 days was recommended.

Magonigle et al. (1975) evaluated the effect of five daily treatments with oxytetracycline hydrochloride at a dose of 22 mg/kg IV on the carrier status of bovine anaplasmosis in eleven 2- to 3-year-old serologically positive cattle. Infection was acquired through natural infection on ranches in Idaho. The duration from infection to treatment is unknown. Carrier status was confirmed by rapid card agglutination (RCA), complement fixation (CF) and inoculation into non-splenectomized calves 8–12 months of age. Carrier clearance was confirmed by inoculating 10 ml of blood from carrier cows into splenectomized calves at 4 and 12 months after treatment. These calves did not exhibit serological, hematological or clinical evidence of *A. marginale* during the 60-day observation period. The results of this study formed the basis for the current OIE recommendation for the clearance of the carrier state. The results of this and subsequent studies are summarized in Table 1.

Roby et al. (1978) reported elimination of the *A. marginale* carrier state with long-acting oxytetracycline (Liquamycin LA 200, Pfizer Animal Health.). Fourteen 12- to 16-

month-old Holstein-Friesian calves were experimentally infected with an unspecified number of parasitized erythrocytes (Virginia isolate) contained in 5 ml of heparinized blood from a known carrier cow. Calves developed clinical anaplasmosis with a rickettsemia between 1% and 10% in 3–4 weeks after infection and recovered spontaneously in 60 days. At 64 days post-infection, the following treatments were applied to groups of four animals: Group A, 20 mg/kg IM twice, 7 days apart; Group B, 20 mg/kg on three occasions, 7 days apart; and Group C, 20 mg/kg on four occasions, 7 days apart. Two animals were treated with 11 mg/kg OTC, IV for 12 days as treated controls. At 83 days after treatment, 80 ml of whole heparinized blood was subinoculated into splenectomized calves to confirm that the carrier cows were free from anaplasmosis.

Kuttler (1980) repeated this experiment using five Holstein-Friesian cows. Three animals were artificially infected with 2 ml carrier blood infected with a Texas isolate and two cows received 2 ml blood infected with a Virginia isolate. Animals developed a parasitemia and illness. Ten months after infection the cows were treated with 20 mg/kg long acting OTC, administered IM twice, 7 days apart. Carrier clearance was confirmed following injection of 50 ml blood into splenectomized calves at 124 days after last treatment.

Magonigle and Newby (1982) successfully cleared carrier infections in fourteen 10- to 15-year-old cows naturally infected with an Indiana isolate of *A. marginale*. Carrier status was confirmed by CF and RCA following infection the previous summer. Cows were treated with 20 mg/kg long-acting OTC administered IM at 3-day intervals for four treatments. Carrier clearance was confirmed by inoculation of 10 ml blood into splenectomized calves at 5 months after last treatment. Calves were monitored for 56 days using RCA and CF.

Kuttler (1983) successfully cleared carrier infections in 6 cows aged approximately 7 years and weighing 630 kg. Three animals were artificially infected with a Texas isolate of *A. marginale* and 3 animals were infected with a Virginia isolate. The inoculation dose and parasitemia is not known. Animals were confirmed CF and RCA positive after infection. At 308 days after infection cows were treated with 20 mg/kg long-acting OTC administered IM on 2 occasions, 7 days apart. Carrier clearance was confirmed by inoculation of 50 ml blood into splenectomized calves at 120 days after last treatment. Calves were CF negative by 52 days and RCA negative by 72 days following treatment.



Swift and Thomas (1983) conducted a carrier clearance experiment using 16 cows of unknown age weighing between 491 and 677 kg at the time of treatment. Cows had acquired infection through natural exposure to *A. marginale* infections in Wyoming. Cows were confirmed CF and RCA positive although the time from infection to treatment was unknown. Cows were divided into two groups of eight cows. The first group received 20 mg/kg long-acting OTC administered IM on three occasions, 3 days apart. The second group received LA OTC at 20 mg/kg IM on four occasions, 3 days apart. Carrier clearance was confirmed by inoculation of 5 ml blood from each animal, pooled and injected into three splenectomized calves at 120 days after last treatment.

Rogers and Dunster (1984) demonstrated clearance of carrier infection in 4 yearling calves artificially infected with 10 ml of 1% parasitized blood obtained from a splenectomized calf infected with a North Queensland (Australia) isolate of *A. marginale*. Parasitemias were confirmed at 7–11 days after infection. Reactions in three cattle were controlled with 3 mg/kg imidocarb, 12 days after infection and in the fourth animal with 10 mg/kg OTC. Animals were confirmed ELISA positive following these treatments. At 73 days following infection animals were treated with 20 mg/kg LA OTC divided between IM and subcutaneous routes. Injections were administered on 3 occasions, 7 days apart. Carrier clearance was confirmed by inoculation of 500 ml blood injected into splenectomized calves at 65 days after last treatment. Calves failed to show signs of anaplasmosis for 93 days after inoculation. The ELISA test used became negative in 20 days after treatment.

Ozlem et al. (1988) reported the successful chemosterilization of 4 cows and 13 young bulls following natural infection with *A. marginale* in Turkey. Eight animals (four Holstein-Friesian cows and four young bulls) had previously shown signs of acute anaplasmosis and the remaining nine cattle appeared in good health but demonstrated parasites on blood smear. The group of acutely infected animals was treated with 20 mg/kg oxytetracycline administered IM on three occasions, 3 days apart. The group of nine asymptomatic cattle was treated with 20 mg/kg on two occasions 3 days apart. The authors claimed that the absence of parasites on blood smear was evidence of chemosterilization. However persistent infection is characterized by sequential rickettsemic cycles ranging from  $10^2$ – $10^7$  parasitized erythrocytes that occur at approximately 5-week intervals. During these

cycles, infected erythrocytes are not always detectable in stained blood smears, but ticks were able to acquire *A. marginale* infection from carrier cattle (Eriks et al., 1989).

In summary, these data suggest that successful clearance of persistent *A. marginale* infections can be established by administering oxytetracycline intravenously to cattle at 11 to 22 mg/kg for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Intramuscular oxytetracycline administrations of 20 mg/kg on 2, 3 or 4 occasions at intervals ranging from 3–7 days were also reported to be effective at eliminating carrier infections (Roby et al., 1978; Kuttler, 1980; Magonigle and Newby, 1982; Kuttler, 1983; Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem et al., 1988).

A major limitation of these previous studies was the dependence on the CF or RCA test to determine seronegativity. This criterion was used in many cases as an indicator of successful chemosterilization. Recent studies have shown that the CF test detects antibodies sooner than the RCA test, but does not detect carriers for as long (reviewed by Potgieter and Stoltz, 2004). Recently a new competitive ELISA test, which uses recombinant *A. marginale* Major Surface Protein 5 (MSP-5), has been developed and marketed in the U. S. (Anaplasma Antibody Test Kit, VMRD, Inc. Pullman, WA; Torioni De Echaide et al., 1998). Bradway et al. (2001) demonstrated that the sensitivity of the CF test was 20% with a specificity of 98%. When this is compared with a sensitivity of 96% and specificity of 95% demonstrated for the cELISA test by Torioni De Echaide et al. (1998), it suggests that previous studies may have wrongly concluded that seronegativity indicated successful chemosterilization.

A second limitation of many of the published reports is the use of isolates obtained from a single location or rarely two locations. Multilocation studies were however confounded by small numbers (Kuttler, 1980). Recent studies using modern molecular diagnostic techniques have identified distinct phylogenetic differences between isolates of *A. marginale* obtained from different geographic locations (de la Fuente, 2001a). Studies have demonstrated that these differences can translate into dramatic differences in the ecology of the disease. de la Fuente et al. (2001b) demonstrated that a Florida isolate of *A. marginale* was not tick transmissible whereas an Oklahoma isolate was. This difference was associated with changes in MSP-1a. Based on these observations it is possible that other phylogenetic

differences exist that may translate into differences in antimicrobial susceptibility (see section 3).

#### ***Tetracyclines administered in feed***

Brock et al. (1959) first reported a study in which fourteen, 2-year-old Hereford cattle were previously experimentally infected with anaplasmosis and therefore assumed to be carriers. Cattle were randomly assigned to one of three treatment groups. The dose rate of CTC was determined by the average weight of the animals in the lot. Cattle in pen 1 received 5 mg/lb (11 mg/kg) CTC daily for 60 days. Cattle in pen 2 received 2.5 mg/lb (5.5 mg/kg) CTC daily for 60 days and cattle in pen 3 received 1.5 mg/lb (3.3 mg/kg) CTC daily for 60 days. A CF test was run at 2 weekly intervals during the treatment period and thereafter monthly for 3 months. Subinoculation of calves with 500 ml blood from each treated animal was conducted at the end of the 60-day treatment period and again 60 days later. Splenectomized calves were monitored for 60 days using the CF test and hematological methods. All animals were found to be CF negative after the 60-day feeding period. All of the calves remained negative for a 60-day period following subinoculation indicating that subinoculated blood was not infectious.

In a second experiment 475 Angus cattle were tested for anaplasmosis using the CF test. Nineteen of the animals were positive to the test. One of these was sold and the remaining 18 were fed 5 mg/lb (11 mg/kg) CTC for 60 days. Fifteen of these were CF negative after 2 months of feeding. No subinoculation tests were conducted to verify that blood from these animals was not infectious. Although these studies were not published in a peer-reviewed journal, the results of the first study appear credible given that blood from each animal was subinoculated twice into splenectomized calves.

Franklin et al. (1965) conducted two experiments in Texas in which 20 anaplasmosis reactor cattle were fed chlortetracycline at 5 mg/lb (11mg/kg) for 30 and 60 days, respectively. Data were compared with a group of 10 known carrier animals, which served as a control group. In the first experiment, 10 animals, weighing 690–990 lbs were fed medicated feed individually for 30 days. Blood samples were collected monthly for 8 months to determine serological status by CF testing. Following treatment, 8 out of 10 cows

demonstrated a decrease in serum antibody titer. Blood from treated cattle was inoculated into intact calves between 6 and 8 months after treatment. All calves remained negative following subinoculation. However it is noteworthy that the method used to determine negativity, the subinoculation dose and the duration of monitoring following subinoculation was not specified. This significantly curtails the inferences that can be drawn from these data.

In the second experiment, 10 reactor cows with an average weight of 896 lbs were hand-fed 11 mg/kg chlortetracycline for 60 days. Five cows were confirmed carriers by subinoculation and the remaining 5 cows originated from an enzootic herd. Blood samples for CF testing were collected monthly for 5 months after treatment. At 120 days after treatment individual intact calves were inoculated with 5ml of blood from each reactor. Seven out of 10 reactors demonstrated a decrease in serum antibody titer at the end of the feeding period. All treated cattle failed to infect intact calves following subinoculation. However the method used to determine this and the duration of monitoring post-inoculation was not specified. Inferences that can be made from these data are also confounded by the fact that animals originated from one locality. Furthermore, in the second experiment, animals were hand fed which facilitates greater control of intakes than occurs when CTC is fed to range cattle.

Franklin et al. (1966) examined two range herds consisting of 600 cattle that were fed various levels of CTC. One herd of approximately 300 head was fed 0.5 mg/lb (1.1 mg/kg) daily for 120 days during the winter. Prior to initiation of the feeding trial 60% of the herd were found to be CF positive. One hundred twenty days after feeding, 8% of the herd was CF positive whereas no animals were found positive on the RCA test. In the second herd of 300 cattle, the herd was divided into groups that were fed 1 mg/lb, 2.5 mg/lb and 5 mg/lb for 30- or 60-day periods. The initial herd was 70–75% positive on the CF test. This study was primarily conducted to measure the agreement between the CF and RCA test. The results indicate that the RCA test was preferable in field situations. Again this paper was not peer reviewed and sterilization of infections was not confirmed by subinoculation into splenectomized calves.

Franklin et al. (1967) conducted a study that examined medium and low level feeding of chlortetracycline with comparison between the CF and RCA test. In the first experiment,

eight intact carrier calves, 8–12 months of age, were fed 2.5 mg/lb CTC per head per day for 45 days. All calves were negative on both the CF and CA tests at 17 days following treatment. All animals were splenectomized at 75 days after the end of feeding. Three calves were not fed any antimicrobial and were designated untreated controls. Splenectomized calves did not demonstrate any signs of infection although the monitoring period was not specified. In a second trial, only 2 carrier calves were fed CTC at 0.5 mg/lb or 1 mg/lb daily for 90 and 41 days, respectively. Both calves were negative to the CF and RCA tests after treatment and remained negative following splenectomy and challenge. Deficiencies in the sensitivity and specificity of these tests make it difficult to comment on whether carrier clearance was actually achieved in these animals. The authors conclude that low level daily feeding of CTC at 0.5–1 mg/lb CTC for 60–90 days respectively can eliminate the carrier state in the non-vector season.

Sweet and Stauber (1978) conducted a regional serological survey followed by oral antibiotic therapy in infected herds in Idaho and southeastern Washington. Of the 3,920 cattle from 31 herds that were tested, 214 (5.5%) were found to positive on either or both the CF and RCA test. One hundred ten carrier cattle identified in this way were segregated and treated by feeding a molasses-barley pellet containing 22 or 110 g/kg oxytetracycline or crumbles containing 4.4 g/kg oxytetracycline. Carriers were treated for 45 days to achieve a daily consumption of 11 mg/kg/day. There was no difference in the effectiveness of the two tetracyclines tested. All 110 animals were found to be seronegative but the paper does not specify how long after treatment this testing was conducted. Goff et al. (1990) observed that application of the antimicrobial interrupted continuity of stimulation of antibody as detected by complement fixation. It may be that these animals were in this refractory period when this testing occurred. Furthermore, this study did not include any subinoculation studies. This study is therefore confounded by the deficiencies in the sensitivity and specificity of the CF test highlighted previously.

### ***Studies failing to establish chemosterilization using published tetracycline protocols***

Kuttler and others (1980) reported the results of a field trial in Texas involving 43 naturally infected cows (see Table 2). *A. marginale* carrier status was identified by CF and

RCA tests and confirmed by subinoculation of 4 ml of blood from groups of three cows into splenectomized calves. Once persistent infections were confirmed, cows were divided into three treatment groups. Group A consisted of 15 cows that received 20 mg/kg LA OTC administered IM, twice, 7 days apart. Group B consisted of 14 cows that received the same dose administered on 4 occasions at 3- or 4-day intervals. Group C consisted of 14 animals that received the same injection on 3 occasions, 7 days apart. Animals were tested at 40 and 90 days after treatment.

At 40 days, 20 ml of blood from nine cows from each group was collected. In each group, three cows were tested individually on a one-to-one basis with splenectomized calves. The blood from the remaining 6 cows was pooled and injected into 2 splenectomized calves. At 90 days after treatment, 20 ml blood from 3 cows in group A and C and 4 cows in group B was inoculated on a one-to-one basis into splenectomized calves. At the end of the study only 4 of the 43 treated cows were negative for anaplasmosis although serological tests suggested a higher number. The authors concluded that the failure in chemosterilization came about due to the active circulation of *A. marginale* infection in the herd at the time of treatment.

Goff et al. (1990) attempted chemosterilization of 52, eight-month-old steers and 13 adult cows naturally infected with a Washington strain of *A. marginale*. Six animals were found to be positive on the CF test and 60 animals were positive using an indirect immunofluorescence test (IIF). One animal was removed from the study. The remaining 64 test animals were identified as positive using a DNA probe. At the end of testing, all animals were treated with 20 mg/kg LA OTC administered IM on four occasions, 3 days apart. One month after treatment, 1 animal was CF positive, 44 animals were IIF positive and 2 animals were identified as positive using the DNA probe. Two months after treatment 2 animals remained CF positive, 17 animals were positive on IIF and samples from 2 new animals hybridized with the DNA probe.

These data suggest that the drug did not eliminate infections in all cattle. Some may have been cleared but in the absence of subinoculation data it would be hard to definitively ascertain successful chemosterilization. In this study the drug did clear the level of infection to below the sensitivity of the DNA probe and interrupted stimulation of antibodies. These

data also indicate that the DNA probe would not be a substitute for using splenectomized calves to determine successful clearance of persistent infections.

The first study to comprehensively question many of the accepted approaches to chemosterilization was conducted by our research group and is presented in chapter 3 of this dissertation (Coetzee et al., 2005). Forty Angus X Simmental steers aged 7 to 9 months at the time of treatment were infected with  $2 \times 10^9$  parasitized erythrocytes obtained from a field case of anaplasmosis in Oklahoma. After the steers recovered from acute infection, seroconverted, and were confirmed infected using nested PCR followed by DNA hybridization, the carrier status of each animal was confirmed by sub-inoculation of blood into a separate, splenectomized Holstein calf.

Carrier steers were blocked by bodyweight and randomly assigned as follows to four treatment groups: Treatment A, 300 mg/ml solution of oxytetracycline (Tetradure LA-300, Merial Canada Inc.) administered at 30 mg/kg, by intramuscular (IM) injection on day 0; Treatment B, the same 300mg/ml solution of oxytetracycline administered at 30 mg/kg, IM on day 0 and again on day 5; Treatment C, a 200 mg/ml solution of oxytetracycline (Liquamycin LA-200, Pfizer Animal Health) administered at 22 mg/kg, intravenously (IV), q 24 h for 5 days (a treatment dose that corresponds with current OIE recommendations for treatment prior to export). The fourth group consisted of untreated infected control cattle. All steers remained positive by nested PCR and cELISA positive at 60 days after treatment. Infection was confirmed by subinoculation of blood into splenectomized Holstein calves. These results demonstrated that all the treatment regimens tested failed to clear *A. marginale* infections in carrier cattle.

### ***Aromatic diamidines and carbanilides***

In contrast with the tetracycline antimicrobials, much less is known about the aromatic diamidines and carbanilides. This is probably due to their limited application to the treatment of protozoal infections in livestock and their high toxicity profile. The action of certain aromatic diamidines on *Babesia canis* was reported by Lourie and Yorke (1939). Some of these compounds have since proven safe in the treatment of bovine babesiosis and, in some cases, anaplasmosis. Diamidines are either aromatic (diminazene aceturate,

pentamidine isethionate, phenamidine isethionate) or carbanilide (amicarbalide, imidocarb dipropionate) (reviewed by Lindsay and Blagburn, 2004). Only diminazene aceturate and imidocarb dipropionate are currently available for use in cattle in certain territories and will be discussed further in this section.

### ***Diminazene aceturate (Berenil, Hoechst)***

#### ***Pharmacology***

Diminazene aceturate was discovered on 16 March 1954 in the research laboratories of Farbwerke Hoechst A.G. (Gummow et al., 1994). For almost half a century it has been an important compound for the treatment of trypanosomiasis and babesiosis. In territories where this compound is approved, it is labeled for the cure and prevention of redwater in cattle and the cure of babesiosis in dogs and horses. It is also labeled for the prevention of *B.a bovis* infection for 2 weeks and *B. bigemina* for 4 weeks (De Vos, 1979). This compound is known to modulate the effect of primary infection, but also allows the development of premunity, which is advantageous in endemic areas (Kuttler, 1988). This feature however precludes the widespread use of this compound in chemosterilization protocols at the usual recommended dose rate.

The exact mechanism of action and *in vivo* behavior of diminazene has only recently been elucidated. Initially it was thought that selective blocking of kinetoplast DNA replication was the major mode of action against trypanosomes (reviewed by McDougald and Roberson, 1988). Newton (1967) reported that diminazene is rapidly and irreversibly bound to calf thymus DNA in a ratio of 1 molecule diminazene for every 4 to 5 DNA nucleotides. This binding was thought to interfere with DNA synthesis as the spacing of the amidino groups of diminazene appeared to be critical to allow the formation of complexes with DNA. Contrary to a previous hypothesis, there was no evidence that diminazene intercalated between adjacent nucleotide pairs. These trials have not been repeated using DNA derived from babesial organisms.

Pilch et al. (1995) conducted a study that demonstrated that diminazene binds to DNA duplexes and to a RNA duplex. DNA binding was accepted to be via complexation into the minor groove of AT-rich domains of DNA double helices. However later research found that



diminazene can bind to RNA as well suggesting intercalative as well as minor groove binding properties. The authors speculate that these findings support previous reports that diminazene may also act by inhibiting topoisomerase enzymes. This activity may prevent DNA supercoiling and decatenation of original chromosomes and replicates.

The pharmacokinetics of diminazene in cattle has been studied over the past 10 years. Gummow et al. (1994) found that a two-compartment model best described the pharmacokinetic behavior of diminazene in cattle. Peak concentrations of diminazene ( $3.24 \pm 0.16 \mu\text{g/ml}$ ) were reached  $49.8 \pm 7.6$  min after intramuscular administration of 3.5 mg/kg diminazene. Diminazene is widely distributed in the body with a volume of distribution ( $V_{d_{ss}}$ ) of 2.1 L/kg. The compound also appears to be very slowly eliminated ( $T_{1/2} = 222\text{h}$ ). This resulted in a mean residence time of 13.27 days. The authors suggested that these data support a pre-slaughter withdrawal period of 66–83 days. This has precluded the registration of this compound in many territories including the European Union and the U.S.

#### ***Chemosterilization of persistent Babesiosis infections with diminazene aceturate***

The clinical application of diminazene therapy has been reviewed by Kuttler (1981). The usual intramuscular dose rate is 3–5 mg/kg but Kuttler found that it was effective against *B. bigemina* at much lower levels. Dosages ranging from 0.5–3.0 mg/kg were highly effective. This study concluded that 0.5 mg/kg was the lowest effective dose. There was no evidence of continued carrier infection at 1–3 mg/kg but this was not confirmed by subinoculation.

Barnett (1965) studied the chemotherapy of *B. bigemina* (Onderstepoort isolate) infection in splenectomized calves as the course of parasitemia in intact calves was thought to be too mild for chemotherapy trials. Four calves in this study were aged 12–18 months. Diminazene was administered subcutaneously as a 7% solution at 1.5 mg/kg, 2.5 mg/kg, 3.5 mg/kg and 5 mg/kg to each of the four calves. The animal treated with 1.5 mg/kg died. At 2.5 mg/kg, animals recovered with no relapses. Following administration at 3.5 mg/kg, blood was cleared of parasites in 24–32 h. The animals that received 2.5 mg/kg and 3.5 mg/kg were immune to challenge at 78 and 109 days, respectively, after treatment. The calf treated with 5 mg/kg was challenged at 68 days after treatment and died from the resulting infection. The

author concluded that a dose of 5 mg/kg resulted in chemosterilization based on the assumption that circulating parasites would have maintained a state of premunity. However, it is impossible to make general recommendations based on a study involving one animal and an expansion of this trial involving a larger number of intact animals has not been done.

Denning (1974) reviewed studies examining the chemosterilization potential of diminazene against babesiosis in cattle. These studies also support the finding that *B. bigemina* responds to lower dose rates than *B. bovis*. Variable chemosterilization was reported after treatment at a dose rate of 5 mg/kg bodyweight. Denning concluded that these findings make it impossible to make recommendations about the dose rate for sterilization of *B. bigemina* infections. He speculated that eradication of *B. bigemina* should be reached at dose rates of 7–10 mg/kg bodyweight. However no data was provided to support this hypothesis.

Dwivedi and Gautam (1977) found that injecting diminazene at 6 mg/kg between 4 and 6 days post-infection with *B. bigemina* resulted in inadequate development of immunity causing animals to be susceptible to reinfection. These results suggest that this dose was sufficient to sterilize the infection in the early stage of disease thereby preventing the development of a preimmunizing carrier state (Sergent, 1965). In a second experiment these authors treated four *B. bigemina* carrier animals with 10 mg/kg diminazene. This dose appeared to sterilize infections based on the failure of 500 ml subinoculated blood from treated animals to infect susceptible splenectomized calves.

For *B. bovis*, Denning (1974) suggested that a dose rate of 7 mg/kg bodyweight was required for chemosterilization. However it is noteworthy that the same review found that dose rates of 10.6 mg/kg were not able to sterilize *Babesia argentina* infections. Subsequent studies have shown that *B. bovis* and *B. argentina* are in fact synonymous, which calls into question the validity of the 7 mg/kg chemosterilization dose rate reported for *B. bovis* (Potgieter, 1978).

#### ***Chemosterilization of Babesia infections with diminazene in other species***

In horses a dose rate of 5 mg/kg administered on two occasions, 24 h apart was found to eliminate *Babesia caballi* infections (Kirkham, 1969; Frerichs and Holbrook, 1974). Dogs

infected with *Babesia gibsoni* were treated with diminazine administered at 11 mg/kg twice, 5 days apart (Fowler, 1972). These animals had demonstrable parasites when they were euthanized 79–93 days after treatment was started. Failure of chemosterilization was demonstrated by the inoculation of an intact dog with blood from a treated animal taken prior to euthanasia. Diminazene is not recommended for administration at dosages in excess of 7 mg/kg due to the risk of CNS toxicity (Kuttler, 1988).

#### ***Use of diminazene against anaplasmosis infections***

In a study conducted by Sharma (1986), the therapeutic and chemoprophylactic effects of diminazene and LA OTC against *A. marginale* were compared. Both compounds were administered at 20 mg/kg, which is almost 6x the recommended dose rate for diminazene. Diminazene was found to have negligible prophylactic effects whereas LA OTC ameliorated subsequent anaplasmosis infections. However, calves treated with OTC or diminazene and oxytetracycline showed normalization in body temperature and a reduction in parasitemia within 7–9 days of treatment. No other reports of chemotherapeutic effects of diminazene against *A. marginale* could be found in the published literature.

#### ***Imidocarb dipropionate (Imizol, Schering Plough Animal Health)***

##### ***Pharmacology***

Imidocarb dipropionate is a carbanilide derivative that has been used for over 30 years in the treatment of bovine babesiosis and anaplasmosis in certain territories (McHardy and Simpson, 1974). It is also used for the treatment of babesiosis in horses and dogs. Schmidt et al. (1969) screened a large number of di-basic compounds against experimental *Babesia rodhaini* infection in mice. Compound No. 3, which was designated as 3,3'-bis-(2-imidazolin-2-yl) carbanilide (4A65) was found to be most effective. This compound was later named imidocarb.

Early formulations utilized the dihydrochloride salt, but this was replaced by the dipropionate salt, which was found to produce less tissue irritation (Roby and Mazzola, 1972; Kuttler, 1988). Imidocarb is usually administered by subcutaneous or intramuscular injection to cattle at a dose rate of 2.1 mg/kg for activity against anaplasmosis. The recommended dose

rate for the treatment of *B. bigemina*, *B. bovis* and *B. divergens* infections is 1–3 mg/kg administered IM or SC (Callow and McGregor, 1970). Intravenous administration is contraindicated (Kuttler, 1981).

The mode of action of imidocarb is uncertain although two mechanisms have been proposed. The first is interference with the production and/or utilization of polyamines and the second is prevention of entry of inositol into the erythrocyte containing the parasite (EMEA, 2001). Bacchi et al. (1981) examined the effect of injecting polyamines into mice infected with *Trypanosoma brucei* and then treated with imidocarb or amicarbalide. These workers found that therapy with carbanilide antimicrobials was highly efficacious without concurrent polyamine administration. However, administration of the polyamine spermadine blocked cures by imidocarb administered at 5 and 10 mg/kg. At doses of 30 mg/kg and higher, spermadine decreased survival time. In an earlier experiment these workers demonstrated that imidocarb and amicarbalide at 250  $\mu$ M inhibited uptake of radiolabelled spermadine by 26% and 29%, respectively (Bacchi et al., 1980). Similar studies have not been conducted using *A. marginale* or *Babesia* organisms. However it is thought that these drugs would act in a similar manner against these organisms.

Pharmacokinetic data and reports on plasma concentrations of imidocarb that may be effective for therapy in cattle are deficient in the published literature. Studies in sheep, goats, dogs and horses have been conducted.

Aliu et al. (1977) conducted a study that evaluated the absorption, distribution and excretion of imidocarb in sheep. Unfortunately the authors did not conduct any pharmacokinetic modeling of these data, which significantly limits the usefulness of this information. In the first part of the study, imidocarb was administered IV at a dose rate of 2 mg/kg to three sheep. A mean initial plasma drug concentration of 10.8  $\mu$ g/ml was recorded which decreased to 1.9  $\mu$ g/ml within 1 h. In the second part of the experiment 4.5 mg/kg was administered IM to seven sheep. A mean peak plasma drug concentration of 7.9  $\mu$ g/ml was achieved within 4 h, which decreased to 4.6  $\mu$ g/ml within the next 2 h.

Trace amounts of imidocarb were still present in the plasma at 4 weeks after administration. The concentration of the drug in the plasma and red blood cells were approximately equal. It was found that 11–17% of the administered IM dose was excreted in

the urine after 24 h. Renal clearance of imidocarb was less than the glomerular filtration rate, indicating net tubular reabsorption. The authors suggest that this process is probably by passive back diffusion rather than by an active transport mechanism. From the 3<sup>rd</sup> day to the 28<sup>th</sup> day, plasma  $T_{1/2}$  was calculated to be 10.9 days. Imidocarb appeared to be between 20.7% and 53.3% protein bound.

Abdullah and Baggot (1983) examined the pharmacokinetics of imidocarb in seven normal dogs and eight goats. An intravenous bolus dose of a 12% imidocarb dipropionate solution was administered at 4 mg/kg. A two-compartment model most closely described the disposition of imidocarb in dogs but in individual goats a three-compartment model was more appropriate. Since imidocarb is a weak base, a comparatively large volume of distribution was observed ( $V_{d_{area}} = 0.432$  L/kg in dogs and 0.544 L/kg in goats). The half-life was determined to be 3.45 h in dogs and 4.18 h in goats.

The larger volume of distribution in goats was thought to be due to passive diffusion of the drug into the rumen fluid where it may become ion trapped. Complete elimination of imidocarb appeared to be prolonged in goats as evidenced by a high ratio of the concentration in the peripheral versus central compartment (6:1) compared with 2.5:1 in dogs. This could be attributed partially to passive reabsorption of unchanged imidocarb from the renal tubules as was reported in sheep. As imidocarb is a weak base, it will be completely ionized in the more acidic urine of dogs and therefore more completely excreted.

Belloli et al. (2002) described the pharmacokinetics of imidocarb dipropionate in horses after intramuscular injection. Eight horses received a single injection of 2.4 mg/kg imidocarb which was followed by blood, fecal, urine and milk sampling. Twelve h after treatment, no detectable plasma concentrations were recorded in any of the treated animals. Disposition of imidocarb conformed to a two-compartment model. The drug appeared to be rapidly absorbed with a mean peak plasma concentration ( $C_{max}$ ) of 0.39  $\mu\text{g/ml}$  attained at 1.16 h ( $T_{max}$ ) after injection. The AUC was 1.02  $\mu\text{g/ml}\cdot\text{h}$  and the elimination half time ( $T_{1/2}$ ) was 5.14 h.

The only published study describing the kinetics of imidocarb in cattle was presented by Good (1998) at the 50<sup>th</sup> meeting of the Joint FAO/WHO Committee on Food Additives (JECFA). In a study which complied with the principles of Good Laboratory Practice, six

lactating cows and eight calves were given a single subcutaneous injection of 3 mg/kg  $^{14}\text{C}$ -imidocarb dipropionate which was formulated as Imizol Injection (Schering Plough Animal Health). A mean peak blood concentration of 1300  $\mu\text{g}$ -equivalents/kg was attained 1 h after treatment and the concentration remained at this level for approximately 4 h. The labeled product was 72–90% bound to plasma proteins. Furthermore, excretion was very slow over the first 10 days. Residues in tissues were also very persistent, particularly in the liver where mean residues of 2200  $\mu\text{g}$ -equivalents/kg were found 90 days after treatment (Ferguson, 1996). These observations are confirmed by other reports suggesting a prolonged retention of the drug in edible tissues (EMEA, 2001). This has restricted the use of this compound in food producing animals in many territories including the U.S.

Adams and Corrier (1980) studied the toxicity of imidocarb dipropionate in cattle. Calves were injected twice, intramuscularly with 0, 5, 10 or 20 mg/kg doses. Transient dosage-dependent signs of toxicity consisted of excessive salivation, serous nasal discharge, diarrhea and dyspnea. Microscopic lesions at high doses included acute severe renal tubular necrosis and focal hepatocellular necrosis. Injection site reactions varied from microscopic areas of necrotizing myositis at the 5 mg/kg dosage to grossly visible areas of necrosis at the 20 mg/kg dosage. All five calves injected with 20 mg/kg died. Mortality did not occur at the 5 or 10 mg/kg dosages. Many of the toxic effects are associated with rapid but reversible inhibition of acetylcholine esterase (Michell et al., 1986).

#### ***Chemosterilization of persistent Babesiosis infections with imidocarb***

Callow and McGregor (1970) were the first to examine the chemosterilization of imidocarb against *B. argentina (bovis)* and *B. bigemina* infections in cattle. Fourteen of 18 steers treated for primary *B. argentina* infections using 0.25–1 mg/kg imidocarb were examined at 7–13 weeks after treatment. All except one steer was shown to be infected. Two calves treated with 0.6 mg/kg and one treated with 0.4 mg/kg imidocarb were examined 1 month after treatment for *B. bigemina* infections. No signs of infection were apparent on blood smear. Following this, 100 ml of blood from each calf was inoculated IV into susceptible splenectomized calves. These transmission tests were negative although the

authors do not specify how animals were monitored or for how long after inoculation this monitoring was conducted.

In a second experiment, these authors established mixed *B. argentina (bovis)* and *B. bigemina* infections in 16 steers. Eight steers were administered 10 mg/kg imidocarb SC. Transmission tests using 500 ml of blood were negative for the eight treated animals but the eight control animals produced mixed infections in the splenectomized calves. In a third experiment these authors studied established *B. argentina (bovis)* infections in 20 steers that had recovered from clinical infections. These were allocated to five groups of four animals per group. Groups were treated with 0.5, 1, 2, 4 or 8 mg/kg imidocarb. On examination all steers treated with 2, 4 and 8 mg/kg were negative but again the authors did not specify after how long, in what way or for what period time animals were monitored.

Roy-Smith (1971) infected splenectomized calves with blood from an apparently less susceptible strain of *Babesia bigemina* in Australia. The origin of this strain was an animal that had been prophylactically treated with 2 mg/kg imidocarb 23 days previously. The splenectomized calves were treated with 0.4 mg/kg imidocarb as described by Callow and McGregor (1970). However in this instance, chemosterilization was not achieved at this dose rate.

Callow et al. (1974a) conducted a series of experiments aimed at evaluating the immunity of animals subsequent to sterilization. In the first experiment, splenectomized calves were exposed to  $2 \times 10^7$  *B. argentina (bovis)* parasites given by subcutaneous injection. Infection was confirmed by the presence of parasites on blood smear. To eliminate infections, imidocarb was administered at doses of 2 mg/kg or greater based on the results of Callow and McGregor (1970). An indirect fluorescent antibody test (IFA) test was used to test for the effectiveness of sterilization treatments. This approach is less than optimal given the shortcomings of many of the earlier diagnostic tests. As a definitive test, brain smears from treated and control animals were examined at 176 days after treatment. Subinoculation of blood into susceptible calves would have been preferable as cerebral location is variable. This was evidenced by the absence of parasites on the brain smear of one of the control animals.

This study was repeated by this research group (Callow et al., 1974b) with similar results. Only blood from animals not receiving imidocarb was subinoculated into splenectomized calves to test persistent infections. It was found that some of these animals had apparently eliminated infections and other had not. Both these studies would have been greatly strengthened by the subinoculation of splenectomized calves to confirm chemosterilization.

Lewis et al. (1981) conducted a study to examine the effect of treatment with imidocarb dipropionate on the course of *Babesia divergens* infections in splenectomized calves. Calves were splenectomized at 3–4 months of age and inoculated 3–5 months later with 2 ml of blood containing  $1.8 \times 10^6$  parasitized erythrocytes. Three groups of three animals were treated on day 7, 14 and 21 after infection. Twenty-one days later, 20 ml blood was taken from each of the three animals treated with imidocarb on day 21, pooled, and inoculated into a splenectomized calf. This process was repeated on day 28 and 35 after treatment. All these splenectomized calves remained free of infection although the authors do not specify how, or for what period time, animals were monitored.

#### ***Chemosterilization of Babesia infections with imidocarb in other species***

Ogunkoya et al. (1981) reported that imidocarb administered once at 5mg/kg bodyweight was found to be highly effective against canine babesiosis caused by *B. canis*. These authors observed that at least 4% of animals treated with this regimen relapsed with babesiosis within 3 months. Adeyanju and Aliu (1982) report a study in which 260 dogs with clinical *B. canis* infections were treated with one injection of imidocarb at 5 mg/kg. Following treatment, 249 dogs recovered, which represents 95.8% of treated cases.

Penzhorn et al. (1995) first reported successful chemosterilization of *B. canis* infections using imidocarb alone or in combination with diminazene. Seven beagle dogs were infected with 2 ml of a whole blood stabilate containing *B. canis* parasites. Following infection dogs were randomly assigned to three treatment groups. Group A consisted of two dogs treated with 7.5 mg/kg imidocarb by subcutaneous injection. Group B consisted of four dogs treated with 3.5 mg/kg diminazene SC followed by 6 mg/kg imidocarb administered SC the next day. Group C consisted of one dog that received trypan blue at 10 mg/kg IV. At 28



days after treatment, 5 ml blood was collected in heparinized tubes from the dogs in group A, pooled and inoculated into a recipient dog. Similarly blood from the four dogs in group B was pooled and subinoculated into a second recipient dog. Fourteen days after first subinoculation the animals were subinoculated for a second time using the same procedure. None of the treated animals in group A and B or recipient animals developed signs of babesiosis during the monitoring period. The dog in group C, however, demonstrated a recrudescence of parasitemia on day 15 after treatment.

Kirkham (1969) reported successful chemosterilization of *Babesia caballi* infections in six horses using imidocarb at 1 mg/lb (2.2 mg/kg) administered on 2 consecutive days. All six horses were cleared of the carrier state as determined by subinoculation into a splenectomized pony. These authors were not able to clear persistent *Babesia equi* infections.

Frerichs and Holbrook (1974) conducted a study using 10 horses infected with *B. caballi*. These were divided into five groups of two horses per group, which were treated with 0.5, 1.0, 2.0, 4.0 and 8.0 mg/kg imidocarb, respectively. Each animal received two injections IM at 24-h intervals. Eight other infected horses were treated with 2 mg/kg imidocarb. Four of these horses were in the acute stage of the disease and four were deemed carriers. One donkey was kept as an untreated control. *B. caballi* was eliminated from all 18 horses as was demonstrated by subinoculation into susceptible horses at 45 days after treatment. Drug reactions were minimal at dosage rates up to and including 2.0 mg/kg.

Frerichs et al. (1973) studied the effect of imidocarb on *B. equi* infections. Three horses and one donkey were assigned to each of three treatment groups. Two horses in each group were proven carriers of *B. equi* by positive subinoculation 30 days previously. The other horse and the donkey in each group were in the early stages of infection. Groups were treated with 4 mg/kg imidocarb on 4 occasions at 24-, 72- and 168-h intervals, respectively. Blood was collected at 18–40 days after final treatment and inoculated into susceptible ponies at a rate of 1 ml/kg. Following subinoculation only one animal in Group A (24-h interval) and one animal in Group C (168-h interval) appeared to be chemosterilized. All animals in Group B (72 hour interval) were negative. All four horses that were still infected after the first trial were isolated for 3 months and re-treated with four injections of imidocarb

administered IM at 4 mg/kg. All these animals were found to be negative on subinoculation after this regimen.

***Chemosterilization of persistent A. marginale infections with imidocarb***

Kuttler (1971) first reported the use of imidocarb for the elimination of *Anaplasma marginale* infections from splenectomized calves. This paper was not published in a peer-reviewed publication. In the first experiment, two calves were each treated with 15 mg/kg imidocarb SC on one occasion. In both cases the animals relapsed at 38 and 35 days after treatment. In a second experiment four calves were treated once daily for 3 days with IM injections of imidocarb. One calf received a dose of 4 mg/kg, two received 5mg/kg doses and one calf received 6 mg/kg. All treated animals failed to show signs of relapsing infections at 90 days after treatment. A subinoculation dose of 200 ml of whole blood was taken at 90 days after treatment from one calf treated with 4 mg/kg and the calf treated with 6 mg/kg imidocarb. This blood was injected into susceptible splenectomized calves, which were monitored for 30 days. Although this study demonstrated that three injections of imidocarb administered at 4 mg/kg at 24-h intervals cleared carrier infections, it is confounded by the fact that it was conducted in splenectomized calves. Furthermore, the sample size of the study was small and the method of assignment to treatment groups was not well defined.

Roby and Mazzola (1972) reported the elimination of *A. marginale* carrier infections with imidocarb. Ten non-splenectomized adult cattle, aged 2–7 years were obtained from an anaplasmosis-free herd. These were infected with 10 ml of heparinized blood from a carrier animal originating from Virginia. Four months to two years later animals were enrolled in the study. All animals were CF positive. Furthermore, 50ml of blood from each animal had also been subinoculated into susceptible splenectomized calves 1 month previously and all animals were demonstrated to be infectious. Two salts of imidocarb were used in this experiment. The first was the dihydrochloride salt, which had previously shown marked swelling and inflammation at the injection site following administration. This reaction was attributed to the high acidity of the aqueous solution of the drug (pH = 3.1). The dipropionate salt had a pH much closer to neutrality (6.5) and therefore caused fewer injection site

reactions. Both salts were injected IM or SC at 5 mg/kg. Where 2 doses were given, the second injection was given 14 days later.

In the first study, five carriers were given a single injection of imidocarb. Two animals received the dihydrochloride salt SC, one animal received the dipropionate salt SC and two received this salt IM. In the second study, five carriers were given two injections of imidocarb, 14 days apart. Three of these received the dipropionate salt IM and the remainder received the dihydrochloride salt. Three carrier animals were maintained under similar conditions to serve as untreated controls.

Three tests were used to determine drug efficacy. Complement fixation (CF) tests were conducted at 30-day intervals as an indicator of infectivity. Thereafter, 50ml of blood was subinoculated into susceptible splenectomized calves at 6 months after treatment. Finally, the spleen was surgically removed from treated cattle that had negative results following the calf inoculation tests. Giemsa-stained blood smears from splenectomized calves were monitored twice a week for 60 days in order to detect the presence of parasites.

The carrier state was not eliminated from the five cattle given a single injection of either salt of imidocarb. The CF test for these five cattle remained positive for 6 months and all five cattle were infective for splenectomized calves. In Group 2, which received two injections of imidocarb 14 days apart, the CF titre became negative between 4 and 6 months after treatment. Susceptible cattle inoculated with this blood did not develop signs of infection on blood smear or CF test. All calves were susceptible to *A. marginale* infection after 60 days. Four of the five treated carriers were then splenectomized and none of them developed signs of anaplasmosis. The non-treated control animals remained positive for the duration of the study. It was also observed that the dipropionate salt caused fewer injection site reactions than the dihydrochloride salt. This study seems to be very well conducted although there were a limited number of experimental animals examined.

Kuttler (1975) examined the use of imidocarb to control anaplasmosis in 469 cattle. Imidocarb dipropionate was injected intramuscularly, twice, 14 days apart at a dose rate of 5 mg/kg. Treatment was deemed "therapeutically effective" based on an initial drop in CF titres noticed after treatment. This was followed by a gradual increase in titer thought to be due to reinfection. One year after treatment the CF level had returned to pretreatment levels.

It is impossible to draw any inferences from this study given that definitive tests to determine the carrier status of animals before and after treatment were not conducted.

### **3. Possible explanations for inconsistent elimination of persistent *A. marginale* infections with tetracycline based protocols**

Successful antimicrobial therapy depends on (1) the susceptibility of the organism to the antimicrobial, (2) achieving adequate drug concentrations at the site of infection, (3) ensuring that drug concentration is maintained for a sufficient duration to be effective, (4) pharmacokinetic parameters of the drug, and (5) the local environment (Bidgood and Papich, 2003). The purpose of this section is to briefly evaluate the chemotherapy of erythrocyte associated organisms in light of these requirements. This section aims to highlight some of the recent molecular advances that may shed some light on the inconsistent results obtained recently with previously published chemosterilization protocols.

#### ***Determining the antimicrobial susceptibility of the organism***

Traditionally pharmacodynamic considerations for bacterial pathogens have been addressed *in vitro* using antimicrobial susceptibility tests such as the Mueller-Hinton broth dilution test or the Kirby-Bauer disk diffusion test. These are readily performed for bacteria given that these can be grown on agar plates in a laboratory. Susceptibility tests facilitate the determination of a minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (NCCLS, 2002). The extension of these techniques to include cell-associated organisms such as *A. marginale* and *Babesia* spp. has been complicated by the need to grow these organisms in cell culture systems.

Up until the late 1970s the normal method for screening compounds for activity against *Babesia* infections was based on testing the compound in laboratory rats or mice infected with *Babesia rodhaini*. Schmidt et al. (1969) used this technique to screen basically substituted carbanilides, which led to the discovery of imidocarb. However most compounds screened against *B. rodhaini* in this way did not give satisfactory results (Irvin and Young,

1977). This was seen as justification to develop an *in vitro* test, which would also reduce the time, cost and numbers of animals involved.

Irvin and Young (1977) first described a technique utilizing whole blood cultures that were incubated overnight in the presence of  $^3\text{H}$ -hypoxanthine. Incorporation of  $^3\text{H}$ -hypoxanthine was measured the next day by liquid scintillation counting. These authors used this system to evaluate the inhibitory effects of imidocarb, amicarbalide and diminazene against *B. rodhaini*, *B. divergens*, *B. major* and *B. ovis*. The first two compounds were found to be most active against *B. rodhaini* and *B. divergens* while diminazene was most active against *B. major* and *B. ovis*. The authors speculate that this system was successful as incorporation of hypoxanthine was essential for nucleic acid synthesis in *Babesia*. This test was therefore a measure of the degree of drug-induced inhibition of nucleic acid synthesis. Preliminary studies were also conducted using diminazene-resistant strains of *B. rodhaini*, which suggested that this system could also be used to determine drug resistant patterns.

Erp et al. (1978) first described a system to culture *B. bovis* in bovine erythrocyte cultures. Blood from parasitized splenectomized calves was incubated at 37° C in a mixture of 50% medium 199 and 50% normal bovine serum. Cells were maintained in suspension by slow stirring and medium was replaced at 24-h intervals. Persistent multiplication of the organism was demonstrated in this system using a short series of subcultures. This suggested that this system was capable of being used for the continuous culture of *Babesia* organisms. This system formed the basis of subsequent techniques that culminated in the development of the microaerophilus stationary phase (MASP) culture technique by Levy and Ristic (1980). The major difference between these techniques was the use of a stationary layer of erythrocytes rather than a suspension culture. This proved to be more convenient given that the constant stirring demanded by the Erp method was not required. This system has since been adapted to perform a variety of screening procedures for potential babesiacides (Nott et al., 1990). Excellent reviews of the cultivation of *Babesia* and *Babesia*-like blood parasites have been published (Kellermann et al., 1988; Schuster, 2002).

Wyatt et al. (1991) described a method for evaluating the growth and viability of cultured intraerythrocytic protozoan hemoparasites using flow cytometry. This assay utilized the selective uptake and metabolic conversion of hydroethidine (HE) to ethidium by live

parasites in intact erythrocytes. The intercalation of ethidium, a DNA binding fluorochrome, into viable parasites allowed the use of fluorescence-activated cell sorting (FACS) to distinguish between erythrocytes containing viable organisms and those containing dead or no parasites. This system was found to correlate very highly with manual counting of the parasite using Giemsa-stained blood films (0.91 for all cell types). Studies with the hemoparasite *B. bovis* utilized this fluorochromasia technique to monitor the effect of diminazene on parasites *in vitro*. As HE conversion and intercalation into DNA is not biased by variations in growth rate or life-cycle stages, the interpretation of these data is simpler than studies evaluating  $^3\text{H}$ -hypoxanthine incorporation. Another major advantage of this system is the speed with which data can be obtained.

Initial attempts to grow *A. marginale* outside the bovine host using cultures of bovine bone marrow, rabbit bone marrow, bovine lymph nodes and tissue derived from the mosquito *Aedes albopictus* were only moderately successful (Hidalgo, 1975; Kessler et al., 1979; McHolland and Trueblood, 1981). Kessler et al. (1979) demonstrated an initial increase in the percentage of parasitized erythrocytes (PPE) within 48 h after the establishment of a whole blood culture of *A. marginale* based on a method used to cultivate *Plasmodia*. The viability of the organism in blood was demonstrated by inoculation of susceptible calves with blood collected from the 13 and 33 day cultures in the first and second experiments, respectively. Kessler and Ristic (1979) also demonstrated the invasion and development of *A. marginale* in noninfected erythrocytes.

Davis et al. (1978) demonstrated the synthesis of DNA and protein by *A. marginale* in bovine erythrocytes during short term culture. This was achieved by examining the incorporation of radio-labeled [ $^3\text{H}$ ] thymidine and [ $^{14}\text{C}$ ] methionine by anaplasmosis in culture. These studies illustrate that *in vitro* cultured anaplasmosis supports many of the metabolic processes that are targets for modern antimicrobials and would therefore facilitate evaluation of these compounds *in vitro*.

In spite of these findings, this whole blood culture system does not support growth of the organism for an extended period of time. Recently Munderloh et al. (1996) cultivated *A. marginale* in a cell line derived from embryos of the tick *Ixodes scapularis*. This system was capable of maintaining an infective isolate for more than 4 years (Blouin et al., 2000).

Blouin et al. (2002) were able to use this system to examine the effect of tetracycline on development of *A. marginale* in cultured *I. scapularis* cells. Various concentrations of tetracycline (0, 0.01, 0.1, 1.0, 5, 10, 20 and 100 µg/ml) were added in medium to cultures 48 h after cell monolayers were inoculated with *A. marginale*. Growth of *A. marginale* was evaluated by indirect ELISA at day 7 post-infection and daily by light and electron microscopy. Tetracycline doses of 5, 10, 20 and 100 µg/ml resulted in significant inhibition of *A. marginale* growth as determined by ELISA. These concentrations also resulted in morphologic deterioration of the organism. Infected cell cultures treated with medium containing 20 µg/ml OTC proved non-infective when inoculated into susceptible splenectomized calves. These data would suggest that the MIC for oxytetracycline against *A. marginale* would be 20 µg/ml. However it is not known how susceptibility determined in a tick cell culture system would correlate with the ability of the drug to inhibit growth of the organism *in vivo*.

Recently a number of studies have demonstrated the successful use of the human promyelocytic leukemia cell line HL-60 to determine the *in vitro* susceptibility of *Anaplasma (Ehrlichia) phagocytophilum* to a number of antimicrobials (Klein, 1997; Horowitz, 2001). This organism is closely related to *A. marginale*, and is reported to be the cause of human granulocytic ehrlichiosis (HGE) (Dumler et al., 2001). A further study using this system demonstrated a difference in susceptibility to chloramphenicol and levofloxacin in *A. phagocytophilum* isolates taken from different geographic locations in the U. S. (Maurin et al., 2003). *In vitro* MICs varied from 0.06–0.5 µg/ml, which is reported to be close to the maximum levels achievable in human serum.

Conflicting reports regarding the success of chemosterilization protocols against anaplasmosis suggest that possible differences in susceptibility between isolates may exist. This hypothesis is supported by the recent identification of two multidrug resistance pumps in the genome of *A. marginale*; although the clinical significance of these pumps has yet to be elucidated (Brayton et al., 2005). In the absence of any other obvious differences between studies that report carrier clearance and studies that failed to clear the carrier state, the concept of resistance should be considered as a possibility. This may be further supported by

the fact that nearly 20 years has passed since many of these “successful” studies were published. This supports further research in this area.

***Challenges associated with achieving adequate drug concentrations at the site of infection***

The treatment of anaplasmosis and babesiosis, where the primary site of infection is within the erythrocyte, is challenging. Sequestration of the organism within the host cells limits drug penetration and, consequently, drug efficacy (Shaw et al., 2001). This effect is compounded in anaplasmosis where the organism is located within an endocytic vesicle in the cytoplasm of the erythrocyte. This would suggest that two barriers to the movement of antimicrobials may exist.

Aliu et al. (1977) found that the concentration of imidocarb in the plasma and erythrocytes of sheep were approximately equal. On the other hand, tetracycline antimicrobials are not actively accumulated in mammalian cells other than those involved in absorption and excretion of the drug. Tetracyclines may however enter cells with altered membrane permeability such as occurs following exposure to polymyxin B or amphotericin B. It has been hypothesized by Scholar and Pratt (2000) that rickettsiae such as anaplasmosis and chlamydiae must be more sensitive to tetracyclines. This is inferred by the fact that these organisms grow within the host cells and are therefore exposed to lower concentrations of tetracyclines than extracellular organisms. However, few studies have succeeded in quantifying the extent to which antimicrobials are taken up by mammalian cells under normal conditions. This would be significant in terms of our understanding of the chemotherapy of intracellular pathogens.

A study conducted by Gabler (1991) investigated the fluxes and accumulation of tetracyclines by human blood cells (RBCs). Doxycycline (Dc) was found to rapidly enter red blood cells with the intracellular concentrations reaching a plateau after approximately 8 min. The ratio between intracellular and extracellular tetracycline concentrations ranged from 2.2–3.4 in favor of the intracellular concentration. This is not surprising given that Dc is highly lipophilic. The addition of 90% serum reduced Dc uptake by 78% but the addition of EDTA restored most of the uptake. The accumulation of three other tetracyclines by RBCs was also studied. In all cases the rank uptake by RBCs was doxycycline > chlortetracycline =



tetracycline > oxytetracycline. The mean intracellular oxytetracycline was estimated to be  $6.1 \pm 2.0 \mu\text{g/ml}$  but in the presence of serum this was reduced to  $0.9 \pm 0.5 \mu\text{g/ml}$ . It can therefore be concluded that tetracyclines can penetrate RBCs but that this uptake is severely reduced in the presence of serum. This raises the question why tetracyclines have proven to be effective against intraerythrocytic pathogens given that the site of infection appears to be partially sequestered from the drug.

It is noteworthy that the presence of anaplasmosis infection significantly reduced erythrocyte membrane acetylcholinesterase activity. This is associated with an increase in osmotic fragility and permeability of erythrocytes. Wallace (1967) conducted a study in six splenectomized calves, 4–5 months old, that were inoculated with 50 ml of blood with a parasitemia of 70%. Erythrocyte AChE activity was assayed following centrifugation and washing of erythrocytes with sodium chloride and standardization of the pH. Two millilitres of acetylcholine bromide was added and the mixture was incubated for 1 h at 25 °C. The AChE activity was expressed as the corrected change in pH/hour with the correlation being made for a negative sample containing water. The results of this study indicated a reduction in AChE activity relative to peak anaplasmosis infection. The concomitant increase in cellular permeability may lead to a greater accumulation of tetracyclines that would occur in the intact erythrocytes. This effect may be enhanced by imidocarb administration, which is characterized by rapid but reversible inhibition of acetylcholine esterase.

Brown et al. (1986) studied blood magnesium values in healthy cattle and in cattle affected with anaplasmosis and eperythrozoonosis. Mean erythrocyte magnesium values were found to increase significantly from previous periods during hemoparasite infection. The mean plasma, serum and erythrocyte magnesium concentrations were correlated to the mean percent parasitemia and the packed cell volume (PCV). The erythrocyte magnesium levels increased significantly after the parasitemia reached 20% and the PCV began to decrease. A strong correlation was observed between the erythrocyte magnesium values and the parasitemia occurring 5 days previously and the PCV occurring 3 days previously.

The observation of increased intracellular magnesium is noteworthy when one considers that tetracycline molecules in the cytoplasm are likely to become chelated with magnesium since the internal pH and divalent metal ion concentrations are higher than those

outside the cell (Schnappinger and Hillen, 1996). Reversible binding of tetracycline through a magnesium ion to the A-site of the 30-S ribosomal subunit blocks the attachment of aminoacyl-tRNA. An increase in intracellular magnesium concentration due to *A. marginale* infection may therefore trap tetracyclines in the cytoplasm of the erythrocyte. This would increase the local concentration of the drug thereby prolonging the attachment of tetracycline to the A-site of the rickettsia. This may also be one of the reasons why tetracyclines appear effective during high parasitemias (when intracellular magnesium is high), but are less effective during the carrier stage when parasitemias are microscopically undetectable and intracellular magnesium concentration is low.

DeLoach et al. (1981) and DeLoach and Wagner (1984) conducted two experiments using imidocarb dipropionate and tetracycline encapsulated in erythrocytes to attempt to increase drug concentrations at the site of infection. In the first study, up to 700 µg imidocarb was encapsulated per ml of bovine carrier erythrocytes with no effect on the carrier cells. About 22 µg of drug was found to bind to 1 ml of cells. Drug loaded carrier cells had an *in vivo* half-life of 28–32 days. Injection of drug loaded carrier erythrocytes at 0.1 mg/kg bodyweight provided cattle with protection against *B. bovis* infection for at least 35 days. This dose rate is 10-fold lower than the usual dose rate for imidocarb. In the second experiment, carrier erythrocytes containing tetracycline were injected into calves and were studied for their pharmacokinetic constants. The half-life of the drug was calculated to be 6.7 h.

Based on these data it is apparent that encapsulating tetracycline in erythrocytes has limited potential for increasing the efficacy of the drug since the elimination of encapsulated and free drug is essentially the same. In the case of imidocarb, however, the use of encapsulated drugs may offer some advantages if the effect is to lower the required dose. Retention of imidocarb in the edible tissue resulting in prolonged meat withhold periods has limited the use of this drug in food producing animals in many territories. This technique may reduce the amount of drug required and therefore reduce the risk to consumers. However, encapsulating erythrocytes is technically demanding and would be difficult to apply to field situations. This would significantly limit the application of this technology.

***Drug pharmacokinetics and challenges to ensure that drug concentration is maintained for a sufficient duration to be effective***

The pharmacokinetics of the various tetracycline formulations was reviewed in section 2. As discussed previously, Blouin et al. (2002) determined the MIC of tetracycline against *A. marginale* in cultured *I. scapularis* cells to be 20 µg/ml. The applicability of MICs derived using this system has not been investigated and these data should therefore be interpreted with caution.

Pharmacokinetic studies in cattle have found that the maximum plasma concentration ( $C_{max}$ ) following IM administration of a long acting formulation containing 200 mg/ml oxytetracycline (OTC) at a dose rate of 20 mg/kg bodyweight was 8.56 µg/ml achieved at 2.53 h after injection. The AUC was 236 µg/ml•hrs and the elimination half time ( $T_{1/2}$ ) was 16.5 h (Clark and Dowling, 2003). Based on these data, plasma concentrations of OTC above the MIC are not attainable following IM administration. The same applies to plasma chlortetracycline (CTC) concentrations following oral administration, which do not peak above 0.2 µg/ml. Theoretically, therefore, it would appear as though neither IM or orally administered tetracyclines achieve adequate serum drug concentrations to be effective against *A. marginale* based on a cell culture derived MIC of 20 µg/ml.

*In vivo* pharmacokinetic studies report a mean oxytetracycline plasma concentration of  $38.5 \pm 6.3$  µg/ml achieved at 1 h following IV administration at 22 mg/kg bodyweight to adult cows (Bretzlaff et al., 1982). This corresponds with the dose rate and route of administration recommended by the OIE. The mean elimination half-life ( $T_{1/2\beta}$ ) was 6.52 h. These data suggest that plasma drug concentrations will decrease to concentrations below an MIC of 20 µg/ml within 7–8 h after administration.

Cunha and Mattoes (2002) state that if the area under the plasma concentration versus time curve (AUC) for tetracyclines approaches 2–4 times the MIC value of the organism, the bacteriostatic effect is maximized. This criterion appears to be met for OTC against *A. marginale* following IV administration although this has not been validated for rickettsial organisms. The second requirement is for plasma OTC concentrations to be above the MIC for at least 50% of the dosing interval for immunocompetent patients, whereas immunocompromised patients require  $T > MIC$  for 100% of dosing interval. The immune

status of *A. marginale* carriers will be discussed in more detail in the next section. However it would appear as if these should be classified as immunocompromised. Under these circumstances, the IV dosing interval would need to be 8 h in order to maintain plasma drug concentrations above the MIC. *In vivo* studies to confirm the validity of this hypothesized dose rate and interval have not been conducted.

***The effect of the local environment, most notably the immune system, on chemosterilization protocols.***

The ability of *A. marginale* to persist in a fully immunocompetent host suggests that persistence involves a mechanism of escape from the immune response. The identification of repeated cycles of rickettsemia, each composed of a progressive, logarithmic increase in organisms followed by a precipitous decrease, led to the hypothesis that persistence reflects the sequential emergence and subsequent immune control of antigenic variants (Palmer et al., 2000).

Distinct outer membrane proteins, termed major surface proteins (MSPs) have been identified on *A. marginale* organisms derived from bovine erythrocytes. These proteins are targets for the host immune response and can also be used in diagnostic assays. MSP1 has been reported to be an adhesin for bovine erythrocytes and tick cells and affects the ability of the organism to be transmitted by ticks (de la Fuente et al., 2002). This protein has been shown to confer partial protection in immunized cattle.

MSP2 and MSP3 are both encoded by large polymorphic multigene families. The MSP2 sequence specifically varies during cyclic rickettsemia. It has been demonstrated that unique MSP2 structural variants arise in each cycle of persistent rickettsemia and that a specific immune response to MSP2 is associated with organism clearance (French et al., 1998). Variation in MSP3 has been demonstrated between geographic locations (Kocan et al., 2000). Antigenic variation in MSP2 and MSP3 occurs by gene conversion of whole pseudogenes and small segments of pseudogenes into a single expression site. This provides an efficient mechanism to generate the large number of variants seen during sequential cycles of persistent infections (Brayton et al., 2003). Little is known about MSP4, but MSP5 has been shown to be conserved on all isolates of *A. marginale* and has proven to be a good

diagnostic antigen for use in a newly developed competitive ELISA test (Torioni De Echaide et al., 1998).

Bacteriostatic antimicrobials by definition arrest bacterial growth and therefore require a competent host immune response in order to facilitate elimination of the infectious agent under treatment. This is especially relevant in the case of the tetracycline antimicrobials where binding to the 30S ribosomal subunit is reversible. The absence of a synergistic relationship between oxytetracycline activity and the host immunity in carrier animals may therefore give rise to the variable success observed in clearing these infections in carrier animals.

Taylor-Robinson and Furr (2000) concluded that successful antibiotic intervention with oxytetracycline in *Mycoplasma* infections depends to a large extent on the ability of the host to mount an adequate immune response. This was demonstrated by contrasting the successful eradication of *Mycoplasma* infections following tetracycline therapy in immunocompetent mice with the failure to eradicate infection following therapy in immune incompetent nude mice.

The importance of an activated cell-mediated immune response (CMI) in clearing the carrier state of anaplasmosis following oxytetracycline therapy was reported by Eckblad et al. (1979) and Lincoln et al. (1982). These workers investigated the effect of administration of a long acting oxytetracycline formulation during the prepatent period of anaplasmosis infection. Each carrier cow was infected with an intramuscular injection of 15 ml of pooled blood from two carrier cows with a 1% and 4% parasitemia, respectively. Oxytetracycline treatment was initiated at a dose rate of 20 mg/kg by deep intramuscular administration with a maximum injection site volume of 10 ml/site. The three treatment groups received treatment on day 7 (T2); day 7 and 14 (T3); and day 7, 14 and 21 (T4) after the day of exposure.

All cows developed evidence of infection as seen by a 0.5% or greater parasitemia and a positive CF and RCA test. The mean prepatent period for groups T1 through T4 was 17, 44, 55 and 62 days, respectively. Although treatment with oxytetracycline did not prevent infection, drug exposure did appear to ameliorate the severity of clinical disease. Given the failure of any of the treatment regimens to clear the carrier state, the authors hypothesized

that a synergism was required between the drug and the acquired immune response. The authors concluded that the administration of oxytetracycline in the prepatent period would suppress growth of the organism, but because the CMI system was not fully activated, the effect was to prolong the incubation period without eliminating infection.

Abbott et al. (2005) recently reviewed the immune response of cattle against persistent *A. marginale* infections. The control of the sequential rickettsemic cycles during persistent infection is associated with development of a variant-specific IgG response, in particular, IgG2 (French et al., 1999). Furthermore, numerous MHC class II-restricted CD4<sup>+</sup> T cell epitopes in the conserved and hypervariable region induce T cell help in order to generate variant specific antibodies. IgG responses appear to be directed primarily against epitopes in the hypervariable region, whereas CD4<sup>+</sup> T cell proliferative and IFN- $\gamma$  responses are directed against multiple epitopes within the conserved and hypervariable region (Brown et al., 2001).

In a study to investigate the stimulation and maintenance of anamnestic responses by specific MSP2 variants to which animals had been immunized, Abbott et al. (2005) describe the rapid and long-term disappearance of CD4<sup>+</sup> T lymphocyte responses specific for *A. marginale* MSP2 in vaccinate animals following challenge with live *A. marginale*. This report suggests that this finding indicates a newly discovered immune modulation whereby antigen-specific T cell responsiveness is lost upon rickettsial challenge. These findings may be significant in terms of our understanding of the relationship between the immune response and the effect of an antimicrobial against persistent *A. marginale* infections. It is apparent that the relationship between chemotherapy with rickettsiastatic antimicrobials and the immune system is extremely complex and requires further examination.

#### **4. Future developments in the field of chemosterilization of persistent rickettsial infections**

Recently a number of studies have demonstrated the successful use of the human promyelocytic leukemia cell line HL-60 to determine the *in vitro* susceptibility of *Anaplasma phagocytophilum* to a number of antimicrobials (Klein et al., 1997; Horowitz, 2001). This organism is closely related to *A. marginale* and is reported to be the cause of human

granulocytic ehrlichiosis (HGE) (Dumler et al., 2001). This system has highlighted the fluoroquinolones as a class of antimicrobial with efficacy against *A. phagocytophilum*. Enrofloxacin is a fluoroquinolone antimicrobial which inhibits bacterial DNA-gyrase (topoisomerase II) and topoisomerase IV (Blondeau, 2004). This activity prevents DNA supercoiling and decatenation of original chromosomes and replicates. The bactericidal activity of enrofloxacin is concentration dependent, with susceptible bacteria cell death occurring within 20–30 min of exposure.

Two published reports indicate that enrofloxacin (Baytril<sup>®</sup>, Bayer Animal Health) is effective against acute *A. marginale* infections at dose rates of 5–10 mg/kg (Schröder et al., 1991; Guglielmone et al., 1996). However the dynamics of the infection following treatment and the potential for elimination of the carrier state has not been assessed. The need for additional trials to study whether enrofloxacin is useful to control severe anaplasmosis infections has been identified (Guglielmone et al., 1996).

Schrevel et al. (1996) recently reviewed new trends in chemotherapy of human and animal blood parasites. These workers highlight a recent study that demonstrated activity of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, Lovastatin and Simvastatin against *in vitro* cultured *Plasmodium falciparum* and *B. divergens* (Grellier et al., 1994). These agents are believed to work on the isoprenoid pathway, which is important for the activation of mitogenic proteins. These agents are currently used in human medicine to treat hypercholesterolemia, but in the future they may be used to potentiate the effect of many existing antimicrobials used in chemosterilization protocols.

In addition to these agents, Schrevel et al. (1996) describe studies examining the effect of microtubular drugs, paclitaxel (Taxol) and its analog, docetaxel (Taxotere), on *P. falciparum* erythrocyte development. These compounds bind to polymerized tubulin stoichiometrically and inhibit depolymerization. These anti-tumor agents may play a role in blocking nuclear division in the parasite. Stich et al. (2004) recently demonstrated that *Anaplasma marginale* assembles an actin filament bundle during intracellular infection. These F-actin appendages are assembled on the cytoplasmic surface of a vacuole containing several organisms. Interestingly, comparison of the Florida, Illinois, St. Maries and Virginia strains revealed substantial polymorphism in the gene encoding the appendage associated

protein. This suggests that while the actin filament bundle may serve as a useful target for antimicrobials, the response to therapy may vary between isolates.

Future advances in molecular biology and x-ray crystallography may allow the identification of novel drug targets resulting in the development of a new generation of antimicrobials. Techniques must be developed to effectively screen these new compounds *in vitro* and ultimately to evaluate efficacious compounds in a representative population of animals. Finally, novel drug delivery systems may be required to optimize exposure of the organism to efficacious plasma drug concentrations.

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**Table 1: Summary of published papers successfully using parenteral oxytetracycline (OTC) to clear the persistent *A. marginale* infections**

Reference	Age/Weight of Carrier	Type of Infection and Source	Carrier Confirmation	Infection to Treatment	Sample Size	Drug Dose (mg/kg)	Route	No. of Treatments	Confirmation of Clearance
Magonigle et al. (1975)	2 to 3 years	Natural (Idaho)	RCA, CF, Inoculation onto NON-SPLENECTOMIZED 8-12 month Calves	Unknown	11	22	IV	Daily for 5 days	10 ml blood into SPLENECTOMIZED calves at 4 and 12 months after treatment (monitored for 60 days); RCA -ve/ CF +ve
Roby et al. (1978)	12 to 16 months (254 to 343 kg)	Artificial (Virginia) 5 ml of carrier blood	Infection 3 to 4 weeks after inoculation (1 – 10% parasitemia) CF POSITIVE	64 Days	4	20	IM	Twice 7 days apart	80 ml blood into SPLENECTOMIZED calves at 83 days after last treatment
					4	20	IM	3 times	
					4	20	IM	7 days apart 4 times	
					2	11	IV	7 days apart Daily for 12 days	
Kuttler (1980)	Holstein-Friesian Cows	Artificial 3 Texas 2 Virginia 2 ml of 1:10 dilution	Parasitemia and illness. Seropositive	10 Months	5	20	IM	Twice 7 days apart	50 ml blood into SPLENECTOMIZED calves at 124 days after last treatment
Magonigle and Newby (1982)	10 to 15 years (494 kg ave)	Natural (Indiana)	Serology (RCA and CF)	Diagnosis in previous summer	14	20	IM	3 day intervals for 4 treatments	10 ml blood into SPLENECTOMIZED calves at 5 months after treatment (monitored for 56 days); RCA -ve/ 1 CF +ve
Kuttler (1983)	7 years (630 kg avg)	Artificial 3 Texas 3 Virginia	CF and RCA positive	308 Days	6	20	IM	2 Injections 7 days apart	50 ml blood into SPLENECTOMIZED calves 120 days after treatment. CF -ve 52 days/ RCA -ve 72 days

**Table 1 (continued)**

Reference	Age/Weight of Carrier	Type of Infection and Source	Carrier Confirmation	Infection to Treatment	Sample Size	Drug Dose (mg/kg)	Route	No. of Treatments	Confirmation of Clearance
Swift and Thomas (1983)	Unknown 491- 677 kg	Natural Wyoming	CF and RCA positive	Unknown	8	20	IM	3 Injections 3 days apart	5 ml blood from each animal POOLED and inoculated into 3 SPLENECTOMIZED calves 120 days after treatment
					8	20	IM	4 injections 3 days apart	
Rogers and Dunster (1984)	Yearling	Artificial Australia	10ml of 1% parasitemia blood inoculated IV. Parasitemias 7-11 days later. Reactions in 3 cattle controlled with Imidocarb 12 days after infection. Remaining animal got 10 mg/kg OTC at 15 days. ELISA positive.	73 Days	4	20	IM/S C	3 Injections 7 days apart	500 ml of blood into SPLENECTOMIZED calves 65 days after treatment. Monitored for 93 days. ELISA negative in 20 days after treatment.
Ozlem et al (1988)	Unknown	Natural Infection (Turkey)	Acute Clinical Infection	Unknown	8	20 mg/kg	IM	3 injections 3 days apart	Parasite negative
			Blood smear	Unknown	9	20 mg/kg	IM	2 injections 3 days apart	

**Table 2: Summary of published papers failing to demonstrate sterilization of persistent *A. marginale* infections using parenteral oxytetracycline (OTC).**

Reference	Age/Weight of Carrier	Type of Infection and Source	Carrier Confirmation	Infection to Treatment	Sample Size	Drug Dose (mg/kg)	Route	No. of Treatments	Confirmation of Clearance
Kuttler (1980)	Cows; Unknown	Natural Texas	Cows identified with CF/ RCA. 4ml of blood from 3 cows pooled and inoculated into a splenectomized calf	Unknown	15	20	IM	Twice, 7 days apart	Tested at day 40 and day 90 using 20 ml of blood. 3 Individual and the rest POOLED blood inoculated into SPLENECTOMIZED calves. ONLY 4/43 CATTLE TESTED NEGATIVE.
					14	20	IM	4 treatments at 3 or 4 day intervals	
					14	20	IM	3 treatments 7 days apart	
Goff et al (1990)	52 x 8 month old steers; 13 cows	Natural Washington	6 CF positive/60 IFAT/ 64 DNA probe positive	Unknown	64	20	IM	4 treatments at 3 day intervals	One month after treatment: 1 CF, 44 IFAT, 2 DNA probe positive. Two months after treatment: 2 CF, 17 IFAT, and 2 new animals DNA positive; 4 apparent treatment failures
Coetzee et al (2005) Chapter 3	Steers 7 – 9 months at treatment	Artificial 2 x 10 <sup>9</sup> organisms Oklahoma	Following artificial infection: PCV, cELISA, PPE, Ht. Inoculated into splenectomized calves. All animals cELISA positive, No visible PPE at time of treatment	65 Days	10	30	IM	Once	cELISA at 30 and 60 days. 50 ml blood into SPLENECTOMIZED calves at 60 days after last treatment. NO CLEARANCE

### **CHAPTER 3. COMPARISON OF THREE OXYTETRACYCLINE REGIMENS FOR THE TREATMENT OF PERSISTENT *ANAPLASMA* *MARGINALE* INFECTIONS IN BEEF CATTLE**

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#### **Abstract**

Anaplasmosis, caused by the tick-borne rickettsia, *Anaplasma marginale*, is an economically important disease of cattle in the United States and worldwide. Cattle that recover from acute infection become carriers in which low or microscopically undetectable *A. marginale* rickettsemia persists. Tetracycline antimicrobials are currently the only drug used in the U.S. for treatment of acute anaplasmosis. There are currently no drugs specifically licensed for elimination of persistent infections. This study tested the efficacy of three oxytetracycline treatment regimens to clear *A. marginale* from cattle that were persistently infected. Forty Angus X Simmental steers, aged 6–12 months were experimentally infected with *A. marginale*. After the steers recovered from acute infection, seroconverted, and were confirmed infected using nested PCR followed by DNA hybridization, the carrier status of each animal was ascertained by sub-inoculation of blood into a separate, splenectomized Holstein calf. The steers were then blocked by bodyweight and randomly assigned as follows to 4 treatment groups: Treatment A, 300 mg/ml solution of oxytetracycline (Tetradure LA-300, Merial Canada Inc.) administered at 30 mg/kg, by intramuscular (IM) injection on day 0; Treatment B, the same 300mg/ml solution of oxytetracycline administered at 30 mg/kg, IM on day 0 and again on day 5; Treatment C, a 200 mg/ml solution of oxytetracycline (Liquamycin LA-200, Pfizer Animal Health) administered at 22 mg/kg intravenously (IV) q 24 h for 5 days (a treatment dose that corresponds with current Office International des Epizooties (OIE) recommendations for treatment prior to export). The fourth group consisted of untreated infected control cattle. All



steers were still nested PCR and cELISA positive at 60 days after treatment. Infection was confirmed by subinoculation of blood into a splenectomized Holstein calf. These results demonstrated that the treatment regimens tested failed to clear *A. marginale* infections in carrier cattle.

## Introduction

Anaplasmosis, caused by the rickettsial hemoparasite, *Anaplasma marginale*, is one of the most prevalent tick-transmitted diseases of cattle and selected ruminants worldwide (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2003). The cost of a clinical case of anaplasmosis in the United States of America has been conservatively estimated to be over US \$ 400 US per animal (Goodger et al., 1979; Alderink et al., 1982). Kocan and others (2003) in a recent review article estimated the cost of anaplasmosis in the USA to be over \$ 300 million per year. Anaplasmosis is currently classified in List B of the Office International des Epizooties (OIE) Terrestrial Animal Health Code due to its socio-economic importance and significance in terms of restrictions in the international trade of animals and animal products (OIE Web site, 2003).

Cattle that recover from acute anaplasmosis remain persistently infected with *A. marginale*. Persistent infection is characterized by sequential rickettsemic cycles ranging from  $10^2$  to  $10^6$ – $10^7$  that occur at approximately 5-week intervals. During these cycles, infected erythrocytes are not always detectable in stained blood smears, but ticks were able to acquire *A. marginale* infection from carrier cattle (Eriks et al., 1989). Thus, carrier animals serve as reservoirs of infection for mechanical transmission and infection of ticks which are biological vectors (Reeves and Swift, 1977; Eriks et al., 1989; Futse et al., 2003).

Oxytetracycline is a tetracycline derivative obtained from *Streptomyces rimosus*. Tetracyclines are bacteriostatic antibiotics that inhibit protein synthesis by reversibly binding to 30S ribosomal subunits of susceptible organisms (Plumb, 2002). Chlortetracycline and oxytetracycline are the only compounds used for treatment of anaplasmosis in the United States (Food and Drug Administration (FDA) Web site, 2003). However, *A. marginale* infections were not cleared from cattle using recommended therapeutic doses (Kuttler et al., 1978; Stewart et al., 1979).

The existence of persistent *A. marginale* infections in cattle despite treatment restricts the movement of animals from areas where the disease is prevalent to areas where the disease is not regarded as endemic such as Canada. Currently, limited movement of feeder cattle from selected Northern States is permitted under the Restricted Feeder Cattle Program (RFCP) (Canadian Food Inspection Agency Web site, 2002). The RFCP facilitates the export of feeder cattle from the U.S.A to Canada between 1 October and 31 March (the non-vector season) provided these animals receive oral tetracycline for 120 days or 2 injections of a long-acting oxytetracycline formulation at a dose rate of 20mg/kg on arrival and 5–7 days later. The Canadian beef industry is looking for alternative treatments, for example a single injection of long-acting oxytetracycline, to reduce the current cost of repeat treatments and the risk of antimicrobial resistance development from long-term use of feed medications. If a single treatment of long-acting oxytetracycline is effective or none of the treatments are effective, then the RFCP may need to be modified accordingly.

The purpose of this study was to compare the efficacy of a single or repeated intramuscular injections of a new long-acting oxytetracycline formulation containing 300mg/ml oxytetracycline (Tetradure LA-300, Merial Canada Inc., Baie d'Urfe, Quebec) with 5 consecutive daily intravenous injections of a 200mg/ml long-acting oxytetracycline product (Liquamycin LA-200, Pfizer Animal Health, Exton, PA) and untreated control cattle.

## **Materials and methods**

This protocol was approved by the Committee on Animal Care (COAC) at Iowa State University.

### **Experimental cattle**

Forty-six Angus and Simmental cross-bred beef steers were initially enrolled in this study to make provision for possible fatalities, but only 40 cattle were eventually selected to enter the treatment phase as study animals. These steers from the Beef Teaching and Beef Nutrition Facilities at Iowa State University were aged approximately 160–230 days and weighed between 214 and 346 kg at the time of selection. Cattle had not received treatment with chlortetracycline or oxytetracycline in the 4-week period prior to study commencement.

The study was conducted in the winter months between 5 November, 2002 and 15 April, 2003, to minimize the risk of vector borne spread of *A. marginale* among test cattle. All cattle were clinically evaluated prior to infection and a blood sample was also taken from each animal for serological screening by a competitive enzyme linked serologic assay (Anaplasma Antibody Test Kit, VMRD, Inc. Pullman, WA) (cELISA) for the detection of *A. marginale* antibodies (Torioni De Echaide et al., 1998). Packed cell volume (PCV) testing was also carried out in order to obtain baseline data and to confirm that animals were uninfected with *A. marginale*.

### **Housing and husbandry**

Cattle were housed in group pens at the Iowa State University Beef Nutrition farm. Following infection and prior to treatment, animals were grouped according to similar size in eight pens of five or six animals per pen. Each pen was 11 m long and 4 m wide with a 4 m bunk space. Following randomization and treatment, the steers were regrouped in pens containing five steers per pen according to their treatment groups. Each treatment group was housed in two immediately adjacent pens and separated from other treatment groups by an empty pen. Drinking troughs were not shared between treatment groups. Pens were bedded with shredded paper, which was replaced as required.

### **Infection with anaplasmosis**

An isolate of *A. marginale* derived from a cattle herd in Oklahoma in 1999 (Blouin et al., 2000) was used in this study. This isolate was genotyped and characterized as being tick transmissible and susceptible to oxytetracycline *in vitro* prior to inoculation (Blouin et al., 2000, 2002). The isolate was preserved in dimethylsulphoxide (DMSO) and maintained as a frozen stabulate in liquid nitrogen prior to inoculation of a splenectomized calf at Oklahoma State University. Three hundred and fifty milliliters of blood was collected from the calf in sterile syringes coated with heparin when the PCV was 34% and the percent parasitized erythrocytes (PPE) were 21.6%. Infection in the test steers was established by inoculating each animal intravenously with 1 ml of blood containing approximately  $2.6 \times 10^9$  infected erythrocytes.

### **Post-infection monitoring**

Following infection, the steers were monitored daily for the clinical signs of anaplasmosis, including anorexia, depression and listlessness. Blood samples were collected at 9, 13, 16, 20, 24, 28, 30, 34, 41, 44 and 62 days post-infection for PPE, PCV and cELISA serology. Body temperature was also measured at selected time points. Blood samples were collected by jugular venipuncture using 18 G, 1 in. needles (Air-tite Products Co. Inc., VA). For serum collection, 10 ml Monoject® No Additive Sterile glass tubes (Sherwood Medical, St Louis, MO) were used. For whole blood (PCV, Hematology and nPCR), 7 ml K<sub>3</sub>EDTA glass tubes (Beckton Dickinson Vacutainer Systems, NJ) were used. Blood in EDTA was refrigerated prior to PCV testing or packaged in insulated material for delivery by overnight courier to Oklahoma State University for determination of the PPE.

Blood smears for PPE determination were stained using a 30-s, three-step staining technique (Hema 3® Staining System, Fisher Scientific) comparable to the Wright-Giemsa method. Two slides were prepared for each blood sample and examined for the presence of *A. marginale* at 100x magnification using a grid. A total of 500 cells were counted within the four squares of the grid and the number of infected cells was recorded. The PPE was the number of infected cells divided by the total number of cells counted, multiplied by 100.

Serologic testing by cELISA was conducted by the Iowa State University Diagnostic Laboratory. The test was conducted in accordance with the method described in the OIE manual of Standards for diagnostic tests and vaccines and the manufacturer recommendations (OIE, 2000; VMRD, 2003). Results are given as percentage inhibition. PCVs were determined by partially filling heparinized capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN) with blood, which were then centrifuged for 10 min using an Adams Micro-Hematocrit centrifuge (Clay Adams, Inc., New York, Model CT 2900) preset to spin blood at a constant rate of 11,500 rpm.

Infected steers were classified as carriers based on the presence of antibodies on the cELISA test and after rickettsemias had fallen below or equal to 1%. After 45 study animals met these criteria, blood from each animal was subinoculated into a separate splenectomized Holstein calf.

### **Splenectomized calves**

The first group of splenectomized calves used to confirm the carrier status of the test system comprised 45 Holstein calves aged approximately 6 to 12 weeks of age and weighing between 45 and 112 kg at the time of surgery. Calves were obtained from the Iowa State University Dairy Breeding Research facility at Ankeny, IA, and a commercial calf rearing facility located in Marshall County, IA. Both facilities are known to have a low incidence of anaplasmosis. Calves were assessed by clinical examination and cELISA to confirm that they had not been previously exposed to *A. marginale* and then splenectomized to increase their susceptibility to *A. marginale* infection.

Splenectomies were performed using the technique described by Thompson et al. (1992). None of the calves received treatment with oxytetracycline in the 4 weeks prior to study commencement. During the 5-day acclimation period and following subinoculation, calves were housed in the livestock infectious diseases isolation facility (LIDIF) at Iowa State University. The calves were divided between three pens in each of three separate rooms. Five calves from a study group were housed together in pens with separate waterers. The room temperature was maintained at approximately 20 °C.

### **Subinoculation of carrier blood**

For each of the 45 steers determined to be cELISA positive with an *A. marginale* rickettsemia <1%, 50 ml blood was collected in a heparinized syringe and inoculated into a splenectomized calf. Filled syringes were stored on ice prior to sub-inoculation which occurred within 8 h of blood collection.

Following inoculation, splenectomized calves were monitored for signs of anaplasmosis by daily observation. Weekly determinations of the PCV were also conducted. The presence of *A. marginale* infection was confirmed by microscopic examination of stained blood smears, an increase in cELISA values, and a PCV below 20%. Calves were euthanized and necropsied once these criteria were met. Based on the PPE results, 5 carrier steers corresponding to the splenectomized calves with the lowest rickettsemia were rejected from the study and remaining steers were then randomized into treatment groups.

**Group assignment and randomization procedures**

Carrier steers were blocked by bodyweight before being randomly assigned to treatment groups in order to ensure a similar weight distribution between groups. Each animal was then randomly assigned to treatment within a weight block by assigning random numbers (Microsoft Excel, Microsoft Corporation).

**Treatment application**

The test formulation of oxytetracycline, Tetradure LA-300, was a sterile long-acting aqueous solution nominally containing oxytetracycline dihydrate equivalent to 300 mg oxytetracycline base per milliliter. A new vial was used on each treatment day. The control antimicrobial, Liquamycin LA-200, was a sterile ready to use solution nominally containing 200 mg oxytetracycline base as oxytetracycline dihydrate per milliliter.

**Tetracycline dose administered**

The test and control formulations were administered at the following dose rates and dosing frequency: Treatment A consisted of a 300 mg/ml solution of oxytetracycline (Tetradure LA-300) administered at a dose of 30 mg/kg, intramuscularly to 10 steers. Treatment B consisted of a 300 mg/ml solution of oxytetracycline (Tetradure LA-300) administered at a dose of 30 mg/kg intramuscularly on day 0 and again on day 5 to 10 steers. Treatment C consisted of a 200 mg/ml solution of oxytetracycline Liquamycin LA-200 administered at a dose of 22 mg/kg, intravenously, once a day for 5 days to 10 steers. Treatment D consisted of 10 untreated controls.

The test formulation was administered to Group A and B by deep intramuscular injection using a 16 gauge x 1.5 in. needle in the left and right neck which is one of the recommended routes of administration. The nominal oxytetracycline content for the formulation and the animal bodyweights obtained immediately prior to administration were used in conjunction with a dosage calculation table to calculate the theoretical dose for each animal. For dosing, a 20 ml hypodermic syringe was used and the required dose was rounded to the nearest whole milliliter with a maximum dose volume of 15 ml per injection site.

The control formulation was administered to Group C by slow intravenous injection into the left or right jugular vein. This is one of the recommended routes of administration. A

16 gauge x 1.5 in. needle was used for each administration. In the presence of swelling or perivascular edema, the contralateral vein was used. In one animal, it was necessary to place a 16 G intravenous catheter into the vein for two administrations. The nominal oxytetracycline content for the formulation and the bodyweights obtained immediately prior to administration were used in conjunction with a dosage calculation table to calculate the theoretical dosage for each animal. For dosing, a 60 ml hypodermic syringe was used and the required dose was rounded to the nearest whole milliliter.

### **Post-treatment monitoring**

Blood samples were taken prior to treatment and at 31 and 60 days post-treatment for direct polymerase chain reaction (PCR), semi-nested polymerase chain reaction (nPCR) and DNA hybridization using the method described by Torioni De Eschaide et al. (1998). Testing was conducted at Washington Animal Disease Diagnostic Laboratory, Washington State University. Briefly, genomic DNA was isolated from 300 µl of blood using a DNA isolation kit (Purogene, Gentra Systems, Inc.) following the manufacturer's instructions. DNA of each sample was resuspended in 100 µl of hydration solution. Primers were designed from the published sequence of *msp5* from *A. marginale* Florida as described by Torioni De Eschaide et al. (1998) and were as follows (5'-3' sequence and location): external forward, 5'-GCA TAG CCT CCG CGT CTT TC-3' (*msp5* positions 254–273); external reverse, 5'-TCC TCG CCT TGG CCC TCA GA-3' (*msp5* positions 710–692); internal forward, 5'-TAC ACG TGC CCT ACC GAG TTA-3' (*msp5* positions 367–387). Two PCR rounds in a final volume of 50 µl consisting of 5 µl of 10x PCR buffer [200 mM Tris-HCl (pH8.4), 0.1 mM KCl ], 1.5 µl of 50mM MgCl<sub>2</sub>, 1 µl dNTPS 10 mM, 1 µl of 20 pmol of each external primers, 1 µl of Taq DNA polymerase [GIBCO, 5 U / µl], 34.5 µl of H<sub>2</sub>O and finally 5 µl of the sample DNA in case of direct PCR or 5 µl of the amplicon from the direct PCR in case of the semi-nested PCR were carried out in a Perkin Elmer GeneAmp 9600 thermocycler. Cycling conditions were preheating at 95 °C for 3 min and 35 cycles of 94 °C for 15 s, 65 °C for 58 s, and 72 °C for 70 s with final extension at 72 °C for 10 min for each round. nPCR was carried out only on samples that were negative for direct PCR. PCR and nPCR products were visualized in a 2% agarose gel following electrophoresis and staining with ethidium bromide.

The treated steers were also tested at 31 and 60 days after first oxytetracycline administration using the cELISA test. A period of 60 days after administration was allowed for clearance of oxytetracycline from the blood in the treated steers prior to post-treatment inoculation of calves.

### **Determination of post-treatment infectivity**

At 60 days post-treatment, approximately 50 ml of blood was collected from each steer and again used to challenge a splenectomized calf per steer using the procedure described previously. The presence or absence of *A. marginale* infection in these splenectomized calves was confirmed as described previously. Calves were euthanized once clinical signs were confirmed and a necropsy was performed on each animal.

### **Statistics**

The mean values and the standard error of the mean (SEM) at each time point for cELISA, body temperature, PPE and PCV were calculated using a Microsoft Excel spreadsheet. Hypothesis tests were conducted using JMP 5 (SAS Institute, Inc., Cary, NC, USA). Analysis of baseline variables was performed using analysis of variance (ANOVA). Subsequent body temperature, cELISA, PCV and PPE results were analyzed using repeated measures analysis. The null hypothesis was that there was no difference between treatment groups. The analysis of variance approach to repeated measures data was used based on a review by Everitt (1995). Multivariate analysis of variance (MANOVA) for repeated measurements over time was selected for these analyses as the assumption of sphericity for univariate tests could not be satisfied. Assuming sphericity in these circumstances would lead to an increase in type 1 error (Everitt, 1995). The sphericity assumption can be regarded as an extension of the homogeneity of variance assumption in independent measures ANOVA. MANOVA makes no assumptions about the form of the covariance matrix of the repeated measures although this method has relatively lower power when the sphericity assumption is valid.

Statistical significance was designated *a priori* as a *P*-value less than or equal to 0.05. The Wilk's lambda test was selected to evaluate within group interactions and evidence of time x group interactions. This test is a likelihood ratio statistic for testing that a multivariate



contrast is zero, assuming multivariate normality and further assuming equality of covariance matrices across groups (Everitt and Dunn, 2001). Where the Wilk's lambda test indicated a statistically significant difference between groups, a one-way analysis of variance (ANOVA) using the Tukey-Kramer HSD method was used to compare the four treatment groups in order to identify which group was statistically different from the rest. Dunnett's method was used to correct the tendency toward type 1 error when comparing treatment groups individually to the control group.

A two-tail Fisher's exact test using a *P*-value of 0.05 to designate significance was used to test whether there was a difference between the number of PCR positive and negative steers in each treatment group. The Fisher's exact procedure calculates an exact probability value for the relationship between two dichotomous variables and is regarded as the gold standard of testing tools for 2 x 2 tables (Ramsey and Schafer, 2002).

## Results

### Carrier Steer Infection Phase of the Study

The results of the infection stage of the study are summarized in Fig. 1 and Table 1.

Prior to inoculation all animals were seronegative for anaplasmosis (<30% inhibition). Nine days after infection the mean cELISA ( $\pm$ SEM) was  $24.82 \pm 2.86\%$  inhibition and 19 animals were classified as positive. Four days later the mean cELISA had risen to  $66.84 \pm 1.55\%$  inhibition with all animals demonstrating evidence of *A. marginale* infection based on a mean PPE ( $\pm$ SEM) of  $0.62\% \pm 0.1$ . At 20 days post-infection the mean cELISA percent inhibition had increased to  $83.92 \pm 0.62$  which coincided with a mean PPE of  $9\% \pm 1.57$  (range: 3–24%). The mean body temperature was  $39.68 \pm 0.17$  at 24 days after infection and the mean PCV was  $23 \pm 0.46\%$ , the lowest recorded during the study. Sixty-two days post-infection, a maximum of one parasitized erythrocyte in 500 cells could be detected in all study animals and the mean cELISA was  $86.29 \pm 1.07\%$  inhibition. The PCV and mean body temperature of all animals was within normal limits. Following assignment to treatment groups these data were retrospectively analyzed and no statistical difference between groups was evident.

### **Post-infection subinoculation of carrier blood into splenectomized calves**

Prior to inoculation on 20 December 2002, all splenectomized animals were seronegative for anaplasmosis (<30% inhibition) and it was assumed that the PPE was zero. Eleven days post-subinoculation, 17 animals were positive on cELISA (>30% inhibition). At 15 days post-subinoculation *A. marginale* intraerythrocytic inclusions were found in all blood smears with a mean PPE ( $\pm$ SEM) of  $16.60 \pm 5.98\%$  (range: 1–75%) and the mean cELISA was  $29.85 \pm 5.98\%$ . Forty-five animals were euthanized approximately 17 days after subinoculation at which time the mean cELISA was  $52.55 \pm 6.17\%$ . All animals demonstrated an increase in cELISA although only 35 were classified as positive. The mean PCV ( $\pm$ SEM) at the time of euthanasia was less than  $15.45 \pm 2.55\%$ . Post-mortem examinations of the subinoculated calves showed gross pathological changes consistent with anaplasmosis infection (Potgieter and Stoltz, 1994). The carrier steers which were used to inoculate the five calves with a PPE of less than 1% were rejected from the study.

### **Treatment phase in carrier steers**

#### ***Post-treatment molecular diagnostic test results***

Prior to the first day of treatment on 10 January 2003, 35 carrier steers were positive for *A. marginale* by direct PCR and all 40 steers were confirmed positive by DNA hybridization and nested PCR.

Thirty-one days post-treatment, 26 animals were positive for *A. marginale* by direct PCR: Seven in Group A, five in Group B, seven in Group C and three in the control group (Group D). The difference between the number of direct PCR positive animals in Groups A and C and Group B, and Group B and D was not significant ( $P = 0.65$ ). There was also no significant difference between groups A and C, and group D ( $P = 0.18$ ). All animals were positive for *A. marginale* on nested PCR and DNA hybridization at this time point.

#### ***Post-treatment competitive ELISA (cELISA)***

Thirty-one days after treatment, the mean cELISA between groups was  $76.47 \pm 1.70\%$  inhibition in Group C and  $88.78 \pm 0.77\%$  inhibition in Group A. There was a significant ( $P < 0.01$ ) difference between the cELISA of Group C and Group A and Group D. None of the other means were different from each other. At 60 days after first treatment there

was no difference between cELISA ( $p = 0.07$ ). Using the Tukey-Kramer HSD method, a significant ( $P < 0.05$ ) difference was found between the cELISA of Group D and Group B. None of the other means were different from each other.

At 90 days after first treatment, there was no significant difference between cELISA ( $P = 0.55$ ).

#### ***Post-treatment subinoculation of blood into splenectomized calves***

The results following subinoculation of carrier blood into splenectomized calves prior to carrier treatment are summarized in Fig. 2 and Table 2.

#### ***Percent parasitized erythrocytes results***

Prior to subinoculation on 11 March 2003, it was assumed that the PPE for all splenectomized calves was zero due to negative cELISA results. At 14 days after subinoculation, *A. marginale* intraerythrocytic inclusions were detected in 35 blood smears. The PPE was monitored at two subsequent time points and at the time of necropsy. The peak PPE recorded over the monitoring period ranged from 1–26% in Group A and 0–33% in Group B. In Group C the peak rickettsemia ranged from 2–45%. Animals in the control group (Group D) had a peak rickettsemia ranging from 2–34%. *A. marginale* organisms were therefore detected in all animals except one animal which died on 27 March 2003. A complete necropsy was conducted on this animal which included bacterial culture and histology. Bacteriological investigation revealed that this animal was suffering from a *Salmonella kentucky* infection presumably exacerbated by the immune suppression caused by splenectomy.

#### **Competitive ELISA (cELISA)**

Prior to subinoculation, the mean group cELISA was negative. Seven days after infection, the cELISA in all animals remained below the positive threshold of 30% inhibition. At 2 weeks after subinoculation, 8 animals demonstrated a cELISA greater than 30% inhibition. By 27 days after subinoculation, 33 animals had been euthanized. All except 1 of the remaining animals had seroconverted. On completion of the study the mean group

cELISA ( $\pm$ SEM) ranged from  $30.3 \pm 9.6$  to  $50.7 \pm 3.9\%$  inhibition. There were no significant differences between groups over time ( $P = 0.08$ ).

### **Packed cell volume (PCV) results**

Prior to subinoculation the mean PCV for all animals was within normal limits (24–46%) (Smith, 1996). All study animals had either died or were euthanized by 35 days after subinoculation. The PCV determined at necropsy in Group A ranged from 6–22% and from 7–30% in Group B. The PCV at necropsy in Group C ranged from 7–16% while animals in the control group (Group D) had a necropsy PCV ranging from 7–22%. There were no significant differences between groups over time ( $P = 0.47$ ).

### **Post-mortem examination**

Post-mortem examinations were conducted on all animals 14–35 days after subinoculation. Most animals showed gross pathological changes consistent with anaplasmosis infection. Hepatomegaly was noted in 30 animals. Twelve animals also had friable livers and 17 livers were mildly to severely icteric or pale. Moderate to severe renal lesions were also noted. Thirty animals were found to have some degree of renal pelvic edema. In 16 animals, multifocal petechial hemorrhages were observed on the kidney surface. Macroscopically, “watery” blood was noted in 36 animals. Petechial hemorrhages were also noted in the epicardium of 19 animals and ecchymoses in the epicardium was observed in six cattle.

### **Discussion**

Our study found that none of the treatment regimens cleared *A. marginale* infection in the persistently infected cattle. These results are contradictory to findings of previous studies in which successful clearance of anaplasmosis carrier infections was achieved using intravenous oxytetracycline dosing regimens ranging from 11 to 22 mg/kg given for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Previous studies reported clearance of the carrier state in cattle using intramuscular oxytetracycline at 20 mg/kg following two, three or four administrations at intervals ranging from 3 to 7 days (Roby et al., 1978; Kuttler, 1980;

Magonigle et al., 1982; Kuttler., 1983; Swift et al., 1983; Rogers., 1984; Ozlem., 1988). Our results are however in agreement with those of Kuttler et al. (1980) and Goff et al. (1990) who report a failure of carrier clearance in naturally infected range cattle using two injections of long-acting oxytetracycline administered at 20 mg/kg by intramuscular injection.

The study design used in our study was comparable with the design of two previous studies. These reported clearance of persistent infections, but were conducted prior to the availability of more sensitive molecular diagnostic techniques and the cELISA test for identification of carrier cattle. Magonigle et al. (1975) evaluated the effect of five daily treatments with oxytetracycline hydrochloride at a dose of 22 mg/kg IV on the carrier status of bovine anaplasmosis in 11, 2 to 3-year-old serologically positive cattle. Carrier clearance was confirmed by inoculating ten milliliters of blood from carrier cows into splenectomized calves at 4 and 12 months after treatment. These calves did not exhibit serological, hematological or clinical evidence of *A. marginale* during a 60-day observation period. The results of this study formed the basis for the current OIE recommendation for the clearance of the carrier state. The major difference between this study and the present study was that the former study incorporated the use of older, naturally infected animals and a considerably longer period between treatment and subinoculation of blood into splenectomized calves. The significance of this in terms of the outcome of this study is not known.

Roby et al. (1978) reported elimination of the *A. marginale* carrier state with two injections of long-acting formulation of oxytetracycline (Liquamycin LA 200, Pfizer Inc.) at 20 mg/kg administered 7 days apart as opposed to the 30 mg/kg dose given 5 days apart in our study. Following experimental infection with an unspecified number of parasitized erythrocytes in 5 ml of heparinized blood from a known carrier cow, the 12 to 16 month Holstein-Friesian calves developed clinical anaplasmosis with a rickettsemia between 1 and 10% and recovered spontaneously in 60 days. This is lower than the rickettsemia recorded in our study which ranged from 3.1% to 24.1% at the peak of infection although the recovery period of 60 days is similar. Furthermore, a longer period of time elapsed (83 days as opposed to 60 days after treatment) before the subinoculation of 80 ml of whole heparinized blood into splenectomized calves to confirm that the carrier cows were free from anaplasmosis.

Tetracycline antimicrobials are bacteriostatic rather than bacteriocidal and the activity of these compounds are believed to be dependant on the time that drug concentrations remain above the minimum inhibitory concentration (MIC) for the target organism. Bacteriostatic antimicrobials arrest bacterial growth. A competent host immune response is usually necessary for elimination of the pathogen. Tetracyclines bind to ribosomes and mRNA and inhibition of protein synthesis is mediated principally through reversible binding with the 30S ribosomal subunit. In bacteria, this binding blocks the attachment of aminoacyl-tRNA to the A site of the mRNA-ribosome complex (Scholar and Pratt, 2000).

Several studies have suggested that oxytetracycline is rickettsiostatic rather than rickettsiocidal against anaplasmosis infections. Eckblad et al. (1979), Lincoln et al. (1982) and Kuttler (1983) demonstrated that administration of oxytetracycline during the prepatent period suppressed multiplication of *A. marginale* and prolonged the prepatent period. Kuttler (1983) concluded that tetracyclines retarded *A. marginale* multiplication rather than actually killing the pathogen. Stewart et al. (1979) observed that the therapeutic dose of long-acting oxytetracycline administered during acute infection was insufficient to eliminate carrier infection. These studies suggest that the immune response to *A. marginale* may not be fully competent during acute infection or that the rickettsemia during this stage overwhelms the ability of oxytetracycline and the immune response to work in concert to eliminate infection.

There may be several reasons why the treatment regimens evaluated in this study failed to eliminate *A. marginale*. Firstly, based on the pharmacodynamics of oxytetracycline it might be hypothesized that the concentrations of oxytetracycline achieved in the present study were not maintained above the MIC for anaplasmosis for a sufficient period of time to eliminate the persistent infection. Blouin et al. (2002) studied the effect of oxytetracycline in vitro on *A. marginale* in a cell culture system using a tick cell line derived from embryonic *Ixodes scapularis*. Tetracycline doses of 5, 10, 20 and 100 µg/ml significantly inhibited growth of *A. marginale* after 7 days of exposure as determined by ELISA and morphological deterioration determined by electron microscopy. *A. marginale* infected cell cultures treated with medium containing 20 µg/ml were non-infective when inoculated into splenectomized calves. In vivo pharmacokinetic studies have found that the maximum plasma concentration (Cmax) following IM administration of a conventional long-acting oxytetracycline

formulation at 20 mg/kg was 8.56 µg/ml achieved at 2.53 h after injection. The C<sub>max</sub> of Tetradure LA-300 was 10.72 µg/ml achieved at 4.64 h after administration (Clark and Dowling, 2003). These results would suggest that plasma concentrations of these formulations of oxytetracycline following parenteral administration may be insufficient to kill the *Anaplasma* organism. This might explain why studies conducted with oxytetracycline given at more frequent intervals demonstrated successful elimination of persistent infections.

Secondly, the location of *A. marginale* during the prepatent period and carrier stage has not been reported and this may be associated with failure to eliminate the organism. It may be that the organisms are not detectable during the prepatent period simply because of the low probability of detecting an infected erythrocyte among the enormous number of non-infected cells. Infected erythrocytes may, however, be sequestered in a “privileged site”, for example the spleen, where oxytetracycline concentrations may be below the minimum inhibitory concentration for the organism (Kuttler, 1983; Potgieter and Stoltz, 1994). Landoni and Errecalde (1992) found that the theoretical area under the tissue concentration vs. time curves (AUC) in the spleen for a period of up to 72 h following intramuscular administration of 20 mg/kg oxytetracycline was 49.47 µg/g h which was in contrast with an AUC of 535.54 µg/g h in the liver and 558.67 µg/g h in the kidney. However, these AUCs were determined from tissue homogenates and therefore assumes that oxytetracycline penetrates all regions of the organ uniformly which may not be the case. The effective AUC specifically against *A. marginale* has not been reported.

The results of the infection phase of the study suggest that the cELISA test results correspond with rickettsemia in the early stages of the disease (Table 1). The first detectable PPEs in this study were concurrent with the first positive cELISA test in all animals and the cELISA peaked at the same time as the peak in the PPE (Fig. 1). The administration of oxytetracycline appears to have reduced circulating rickettsemia in the carrier animals. A significant decrease in cELISA was seen in the group treated with five injections of oxytetracycline (Group C) when compared with the control group and the group treated with only one injection of oxytetracycline (Group A). Further testing involving molecular methods is required in order to establish the extent to which oxytetracycline administration influences antibody response.

It is noteworthy that in both phases of the study involving splenectomized calves, a positive (>30%) cELISA test was not detected in all calves, although they were parasitemic and experienced a notable decrease in the packed cell volume consistent with acute anaplasmosis. Antibodies may have been bound to organisms in the acute phase of the infection or decreased antibody levels may have been a result of immunosuppression resulting from splenectomy. Furthermore, the cELISA was validated on adult cattle and the test was not used to screen *A. marginale* antibodies in splenectomized calves. Our protocol also required animals to demonstrate a change in cELISA level and not necessarily a positive cELISA test. The presence of organisms and positive molecular diagnostic techniques should therefore be regarded as the definitive test for confirmation of *A. marginale* infection in splenectomized calves.

The results of this study demonstrated that subinoculation of blood from all except one of the carrier steers treated with one of the three regimens of oxytetracycline resulted in infection and clinical anaplasmosis in splenectomized calves. The splenectomized calf in which infection could not be confirmed did, however, die in the early stages of the study and it may be possible that it was still in the prepatent period. Administration of a 300 mg/ml solution of oxytetracycline (Tetradure LA-300) administered at a dose of 30 mg/kg, intramuscularly once or twice 5 days apart was not effective for elimination of the persistent *A. marginale* in beef cattle. Furthermore, the study demonstrated that the current recommended OIE treatment protocol of five injections of a 200 mg/ml solution of oxytetracycline administered at a dose of 22 mg/kg intravenously was also not effective for elimination of persistent *A. marginale* infection.

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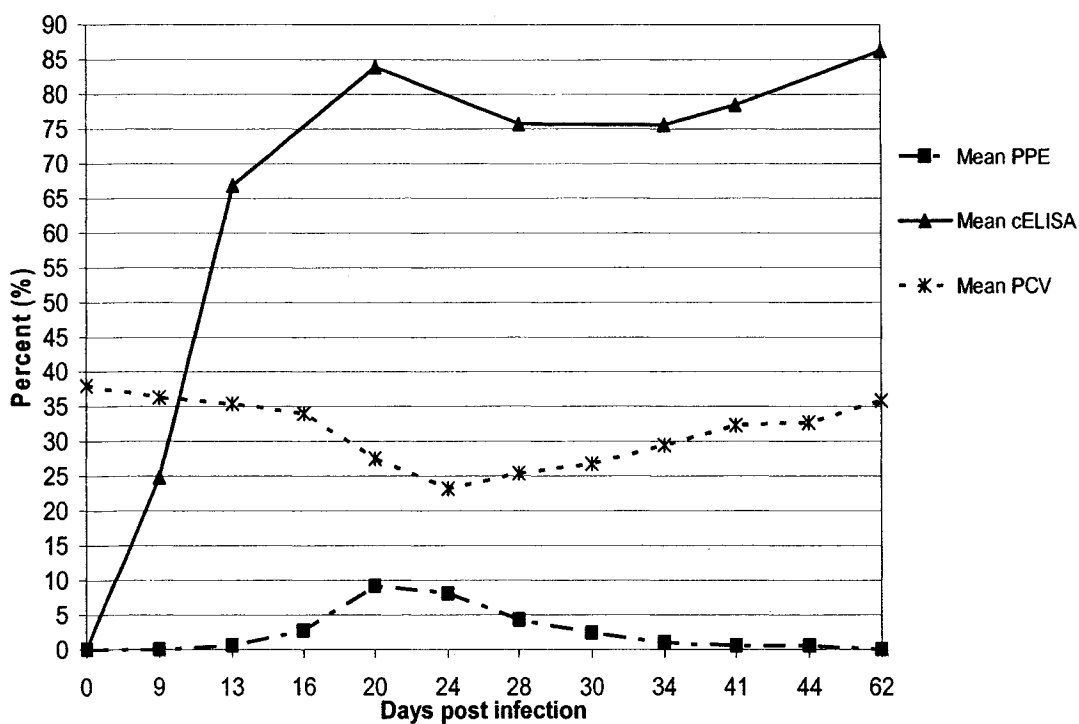
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**Fig. 1: Summary of mean clinical parameters following carrier steer infection with  $2.6 \times 10^9$  *Anaplasma marginal* (Oklahoma Isolate) parasitized erythrocytes**

PCV: Packed Cell Volume; cELISA: Competitive Enzyme Linked Immunosorbent Assay; PPE: Percent Parasitized Erythrocytes

**Table 1: Summary of Mean Clinical Parameters<sup>a</sup> Following Carrier Steer infection with  $2.6 \times 10^9$  *Anaplasma marginale* (Oklahoma Isolate) Parasitized Erythrocytes**

Days Post Infection	0	9	13	16	20	24	28	30	34	41	44	62
PPE (%)	0.00	0.00	0.62	2.72	9.17	8.17	4.31	2.46	1.01	0.55	0.59	0.00
Standard Error	0.00	0.00	0.10	0.41	1.57	1.77	1.40	0.81	0.27	0.14	0.16	0.00
MANOVA P value = 0.74												
Wilkes Lambda Test P = 0.62												
cELISA (% inhibition)	0.00	24.82	66.84	N/A	83.92	N/A	75.71	N/A	75.56	78.45	N/A	86.29
Standard Error	0.00	2.86	1.55	N/A	0.62	N/A	0.69	N/A	0.85	0.85	N/A	1.07
MANOVA P value = 0.47												
Wilkes Lambda Test P = 0.94												
Body Temperature (°C)	39.21	39.19	39.03	38.75	39.56	39.68	38.95	38.86	39.11	38.95	N/A	N/A
Standard Error	0.12	0.11	0.12	0.174	0.154	0.166	0.188	0.124	0.094	0.106	N/A	N/A
MANOVA P value = 0.63												
Wilkes Lambda Test P = 0.31												
PCV (%)	38.03	36.35	35.43	34.00	27.58	23.23	25.40	26.78	29.43	32.35	32.75	35.83
Standard Error	0.46	0.41	0.41	0.41	0.47	0.46	0.63	0.67	0.58	0.59	0.56	0.43
MANOVA P value = 0.14												
Wilkes Lambda Test P = 0.81												

<sup>a</sup>PCV: Packed Cell Volume; cELISA: Competitive Enzyme Linked Immunosorbent Assay; PP Percent Parasitized Erythrocytes

**Table 2: Summary of Mean Parameters taken from Splenectomized Calves (Phase 2) Subinoculated with 50 ml of Blood from Carrier Steers 60 days after Treatment with Oxytetracycline**

Date of Necropsy	Days from Inoculation	Start PCV <sup>a</sup>	End PCV <sup>a</sup>	Start cELISA <sup>a</sup>	Peak cELISA <sup>a</sup>	Mean PPE <sup>a</sup>
Group A <sup>b</sup>	19.2	29.5	11.9	2.0	40.3	6.9
Standard Error	2.7	3.3	2.0	1.7	6.0	2.3
Group B	22.3	32.6	13.5	0.0	30.3	15.6
Standard Error	1.5	0.9	2.2	2.9	9.6	3.8
Group C	22.5	32.8	11.4	3.6	45.9	16.2
Standard Error	1.6	0.7	1.0	3.4	7.5	4.7
Group D	21.1	33.3	12.8	4.0	50.7	12.3
Standard Error	1.0	0.7	1.5	2.5	3.9	3.3
ANOVA P Value	0.9					0.5
MANOVA P value		0.47		0.08		

<sup>a</sup>PCV: Packed Cell Volume; cELISA: Competitive Enzyme Linked Immunosorbent Assay; PPE: Percent Parasitized Erythrocytes

<sup>b</sup>Group A: 300 mg/ml solution of oxytetracycline (Tetradure LA-300) administered at a dose of 30 mg/kg, by intramuscular injection.

Group B: 300 mg/ml solution of oxytetracycline (Tetradure LA-300) administered at a dose of 30 mg/kg by intramuscular injection on day 0 and again on day 5.

Group C: 200 mg/ml solution of oxytetracycline (Liquamycin LA-200, Pfizer Animal Health) administered at a dose of 22 mg/kg, intravenously, once a day for 5 days.

Group D: Untreated infected controls.

## CHAPTER 4. EVALUATION OF THE EFFECT OF SELECTED ANTIMICROBIALS ON *ANAPLASMA MARGINALE* USING FLOW CYTOMETRY

A paper to be submitted to Veterinary Parasitology

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### Abstract

*Anaplasma marginale* is one of the most prevalent tick-borne pathogens of cattle worldwide. Cattle recovering from acute infection remain persistently infected despite treatment with tetracyclines. These carrier cattle serve as reservoirs of infection for mechanical spread and infection of ticks. In this study we used flow cytometric analysis to evaluate the effect of antimicrobials against *A. marginale* in short-term whole erythrocyte cultures. Erythrocytes infected with the Virginia (VGN) or Oklahoma (OK) isolates of *A. marginale* were tested with doubling dilutions of oxytetracycline (OTC), imidocarb (IMD) and enrofloxacin (ENRO). Parasite viability was assessed by FACS for 7 days using the vital dye hydroethidine (HE) which, when converted to ethidium bromide (EB) by live parasites, is detectable by FACS. After 7 days, infectivity of selected cultures was determined by inoculating 400 µl of packed erythrocytes intravenously into Holstein calves. The FACS data were analyzed statistically by multivariate analysis of variance (MANOVA) and the Tukey-Kramer HSD (honest significant difference), as well as Wilcoxon rank sum tests and student t tests for comparison of the two isolates. Receiver operating characteristic (ROC) analysis was used to compare FACS results with culture infectivity. Enrofloxacin inhibited *A. marginale* in a concentration dependent manner, while higher concentrations of imidocarb were less effective than lower concentrations in reducing the number of viable organisms. Oxytetracycline was found to be the least efficacious antimicrobial in this culture system. Differences between isolates were evident at some dilutions. Cultures of erythrocytes infected with the Oklahoma isolate exposed to 4.0 µg/ml enrofloxacin and those of the Virginia and Oklahoma isolates exposed to 1.0 µg/ml appeared to be sterilized. This study



appears to be the only *in vitro* study demonstrating the anti-*A. marginale* microbial effect of enrofloxacin. Flow cytometry proved to be useful for screening antimicrobial activity of drugs against bovine erythrocytes infected with *A. marginale*.

## Introduction

Anaplasmosis, caused by the rickettsial hemoparasite, *A. marginale*, is the most prevalent tick-transmitted disease of cattle worldwide (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2003). Prior to the development of imidocarb dipropionate and the tetracycline antimicrobials, a variety of chemotherapeutic agents, including arsenicals, antimalarials, antimony derivatives and dyes, were used to treat acute anaplasmosis. These compounds had little if any chemotherapeutic effect (Potgieter and Stoltz, 1994). Chlortetracycline and oxytetracycline are the only compounds available for prevention or therapy of acute anaplasmosis in the United States. *Anaplasma* infections are not sterilized at the usual recommended therapeutic doses of the tetracycline drugs (Kuttler and Simpson, 1978; Stewart et al., 1979). There are currently no antimicrobials labeled for the elimination of persistent infections in carrier animals.

Successful antimicrobial therapy depends on (1) achieving adequate drug concentrations at the site of infection, (2) ensuring that drug concentration is maintained for a sufficient duration to be effective, (3) the susceptibility of the organism to the antimicrobial, (4) pharmacokinetic parameters of the drug, and (5) the local environment (Bidgood and Papich, 2003). Susceptibility tests facilitate the determination of a minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (NCCLS, 2002). The extension of these techniques to include cell associated protozoa such as ehrlichia and rickettsia has been complicated by the need to grow these organisms in cell culture systems.

Initial attempts to grow *A. marginale* outside the bovine host using cultures of bovine bone marrow, rabbit bone marrow, bovine lymph nodes and tissue derived from the mosquito *Aedes albopictus*, were only moderately successful (Hidalgo, 1975; Kessler et al., 1979; McHolland and Trueblood, 1981). Kessler et al. (1979) established a whole blood culture of

*A. marginale* based on a method used to cultivate *Plasmodia*. The viability of the organism in blood was demonstrated by inoculation of susceptible calves with blood collected from the 13 and 33 day cultures.

Wyatt et al. (1991) described a method for evaluating the growth and viability of cultured intraerythrocytic protozoan hemoparasites using flow cytometry. This assay utilized the selective uptake and metabolic conversion of hydroethidine (HE) to ethidium by live parasites in intact erythrocytes. The intercalation of ethidium, a DNA binding fluorochrome, into viable parasites allowed the use of fluorescence-activated cell sorting (FACS) to distinguish between erythrocytes containing viable organisms and those containing dead or no parasites. Studies with the hemoparasite *Babesia bovis* utilized this fluorochromasia technique to monitor the effect of parasitocidal drugs on parasites *in vitro*.

The purpose of this study was to utilize whole blood culture and flow cytometric analysis (FACS) to evaluate the effect of oxytetracycline, imidocarb and enrofloxacin against *A. marginale* obtained from two different geographic locations. The percent reduction in HE positive cells was used to select cultures to inoculate into calves in order to determine a minimum rickettsiocidal concentration (MRC).

## **Materials and methods**

This protocol was approved by the Committee on Animal Care (COAC) at Iowa State University.

### **Propagation of *A. marginale* in splenectomized calves**

Two Holstein calves were obtained from the Iowa State University Dairy Breeding Research facility at Ankeny, IA. Animals were confirmed free of *A. marginale* antibodies by competitive enzyme linked immunoabsorbent assay (<30% inhibition) (cELISA Anaplasma Antibody Test Kit, VMRD Inc., Pullman, WA) (Torioni De Echaide et al., 1998). Splenectomies were performed when calves were approximately 3 months old using the technique described by Thompson et al. (1992).

Approximately 6–8 weeks after splenectomy, one calf was infected with 10ml of blood stabilate containing an Oklahoma isolate with 31.6% parasitized erythrocytes. The

second calf received 10ml of blood stabilate containing a Virginia isolate with 35.5% parasitized erythrocytes. Blood stabilates were prepared from packed erythrocytes washed in phosphate-buffered saline (PBS) and frozen in liquid nitrogen at a 1:1 ratio with PBS containing 10% dimethylsulphoxide (DMSO). Stabilates were maintained frozen in liquid nitrogen and dry ice prior to inoculation.

### **Post-infection monitoring**

Following infection, blood samples were collected at least once a week post-infection to determine percent parasitized erythrocyte (PPE) and packed cell volume (PCV). Blood in EDTA was refrigerated prior to PCV testing or packaged in insulated material for overnight delivery by courier to Oklahoma State University for determination of the PPE.

Blood smears for PPE determination were stained using a 30-s, three-step staining technique (Hema 3® Staining System, Fisher Scientific) comparable to the Wright-Giemsa method. Two slides were prepared for each blood sample and examined for the presence of *A. marginale* at 1000x magnification using a grid. A total of 500 cells were counted within the 4 squares of the grid and the number of infected cells was recorded. The PPE was the number of infected cells divided by the total number of cells counted, multiplied by 100.

PCVs were determined by partially filling heparinized capillary tubes (Chase Scientific Glass Inc., Rockwood, TN) with blood which were then centrifuged for 3 min using an Adams Micro-Hematocrit centrifuge (Model CT 2900, Clay Adams Inc., New York).

### **Preparation of erythrocyte cultures**

At the time blood was collected aseptically, in heparin from splenectomized calves, the PPE was 28.6% and 31.4% for animals inoculated with the Virginia and Oklahoma isolate respectively. Whole blood was centrifuged at 600 x g at 4 °C for 15 min and the plasma and buffy coat was removed. Cells were resuspended and washed twice in RPMI 1640 culture medium (Cellgro, Mediatech Inc., Herndon, VA) at 4 °C (Kessler et al., 1979).

Drug susceptibility assays were performed in 96-well culture microplates in the presence of a final erythrocyte concentration of 10% (vol/vol) and serial dilutions of drugs. Forty-eight wells on each plate were inoculated with the Virginia isolate with the remainder

inoculated with the Oklahoma isolate. Cell culture medium comprised RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 25 mM HEPES buffer, 200 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Controls consisted of plates containing erythrocytes infected with each isolate without the addition of the antimicrobials to be tested and non-infected bovine erythrocytes.

### **Antimicrobial agents**

Stock solutions of antimicrobials at a concentration of 1 mg/ml were prepared in cell culture medium. These stock solutions were used to prepare the following antimicrobial dilutions: Oxytetracycline hydrochloride (OTC) (Sigma-Aldrich, St Louis, MO) at 1, 2, 4, 8 and 16 µg/ml; imidocarb (IMD) (N,N'- Bis[3-(4,5-dihydro-1H-imidazol-2-yl)phenyl]urea dipropanoate, 99%) (Sigma-Aldrich, St Louis, MO) at 0.25, 0.5, 1, 2 and 4 µg/ml and enrofloxacin (ENRO) (Fluka, Buchs, Switzerland) at 0.25, 0.5, 1, 2 and 4 µg/ml. For each drug dilution and isolate combination, a volume of 180 µl of each dilution was added to each of 48 wells. Following addition of 20 µl of washed packed erythrocytes, cultures were incubated at 37 °C and 5% CO<sub>2</sub> for 7 days.

### **Cell preparation and hydroethidine staining**

On each study day, 3 wells from each isolate and drug concentration including the control cultures were washed in separate centrifugation tubes containing 1 ml 1x phosphate-buffered saline (PBS). Samples were pelleted by centrifugation for 5 min at 450 x g at 4 °C.

Cell staining for FACS was performed using a method similar to that described by Wyatt et al. (1991). Briefly, dihydroethidium (hydroethidine) (HE) (Molecular Probes, Eugene, OR) was solubilized in anhydrous DMSO at 1 mg/ml as a stock solution. Fifteen micrograms of solubilized HE was diluted in 1 ml PBS which was added to the cell pellet. Suspensions were incubated in the absence of light for 30 min at 37 °C, 5% CO<sub>2</sub>. Following incubation, 980 µl of suspension was removed and replaced with 1 ml PBS. Diluted samples were centrifuged for 5 min at 450 x g at 4 °C. Pelleted erythrocytes were resuspended in 1 ml FACS buffer containing 0.1% bovine serum albumin and 1 x PBS. Unused HE in DMSO was frozen at -20 °C in 1 ml aliquots and thawed at 37 °C prior to use.

### **Flow cytometric analysis**

Cell suspensions to be analyzed by FACS were diluted with PBS to obtain a flow rate of 2000–5000 fluorescent events/s. A total of 150,000 events were counted daily for each of the three wells harvested from culture. An inclusion gate was set based on the forward scatter and side scatter characteristics of the HE-treated uninfected erythrocyte control. The suspensions were analyzed by argon-ion laser fluorescence excitation at 488 nm and emission at 585 nm (range: 563–607 nm) (FL-2) in log Fl 2 data mode using a Becton-Dickinson FACScan and CellQuest computer software (BD Biosciences, San Jose, CA). Fluorescent profiles were recorded for later analysis using FlowJo computer software (Tree Star Inc., Ashland, OR).

### **Testing infectivity of selected cultures**

The infectivity of parasitized erythrocytes following 7 days of culture was determined from samples selected on the basis of the FACS results. The contents of 20 wells (4,000  $\mu$ l) from each selected sample were pelleted by centrifugation at 450 x g for 5 min. Approximately 400  $\mu$ l packed cells were resuspended in 1.5 ml RPMI 1640. Samples were stored on ice prior to inoculation into non-splenectomized Holstein calves.

Fifteen Holstein calves were confirmed free of *A. marginale* antibodies by two consecutive cELISA tests (Anaplasma Antibody Test Kit, VMRD Inc., Pullman, WA). Resuspended erythrocytes were inoculated intravenously by jugular injection using a 16 G, 1 in. needle. Two calves received untreated infected control culture suspensions while one calf received the uninfected control suspension. The remaining 12 calves received selected treated samples. Four calves were randomly assigned to each antimicrobial treatment with two pairs receiving the Oklahoma and Virginia isolate, respectively.

Following inoculation, calves were monitored daily for clinical signs of anaplasmosis, including anorexia, depression and listlessness. Blood samples were collected at least once weekly for approximately 8 weeks post-infection for PPE, PCV and cELISA serology as previously described. After 8 weeks calves failing to demonstrate signs of anaplasmosis were splenectomized and monitored for an additional 4 weeks. Cultures exposed to antimicrobial concentrations that failed to cause disease following subinoculation into calves were deemed

sterilized. These drug concentrations were considered rickettsiocidal after 7 days. Those cultures in which infectivity was retained were deemed treatment failures.

### **Statistical analyses**

Data were entered into a spreadsheet program (Excel 2003, Microsoft Corporation, Redmond, WA) for subsequent calculation and manipulation. The mean  $\pm$  SEM (standard error of the mean) HE positive cell count was calculated from the 3 wells from each culture analyzed each day. The percent reduction in HE positive cells was determined as the mean number of HE positive (infected) cells in the test culture subtracted from the mean number of HE positive (infected) cells in the control culture divided by the total number of HE positive control cells, multiplied by 100.

Hypothesis tests were conducted using JMP 5.1.2 analytical software (SAS Institute Inc., Cary, NC). FACS data were analyzed using repeated measures analysis. The null hypothesis was that there was no difference between treated and control cultures. The analysis of variance approach to repeated measures data was used based on a review by Everitt (1995). The Wilk's lambda test was selected to evaluate within group interactions and evidence of time  $\times$  group interactions. This test is a likelihood ratio statistic for testing that a multivariate contrast is zero, assuming multivariate normality and further assuming equality of covariance matrices across groups (Everitt and Dunn, 2001). In all analyses the Wilk's lambda test indicated a statistically significant interaction ( $P < 0.001$ ).

Accordingly, differences between antimicrobials and antimicrobial dilutions were analyzed using ANOVA and the Tukey-Kramer HSD (honest significant difference) method for multiple comparisons. This test is an exact alpha-level test if the sample sizes are the same and conservative if the sample sizes are different (Hayter, 1984). Statistical significance was designated *a priori* as a  $P$ -value less than or equal to 0.05. Differences between isolates exposed to a particular drug concentration on a particular day were analyzed using Wilcoxon rank sum tests (chi square approximation) as these data were not normally distributed. Pooled differences between isolate means over the 7-day period of the study were analyzed using student  $t$  tests.

Results of cELISA testing following inoculation of selected cultures into Holstein calves were analyzed using a simple linear regression model (Mutapi and Roddam, 2002). In this model the cELISA results were taken as the response variable and days post-inoculation were the explanatory variable. The null hypothesis was that cultures exposed to antimicrobials were not infective and accordingly did not elicit a serological response following inoculation. This hypothesis was tested using a student t test of the slope of the linear regression line to determine if the gradient was zero (Zou et al., 2003). A *P*-value  $>0.05$  indicated that the slope of the regression line could be zero suggesting lack of seroconversion. A statistically significant *P*-value ( $<0.05$ ) indicated the antimicrobial dilution tested did not sterilize infection in culture.

In order to determine the percent HE positive cells which most closely correlated with sterilization of anaplasmosis infections in culture, we conducted receiver-operating characteristic (ROC) analysis on each day of the study (Greiner et al., 2000). The underlying assumption of ROC analysis is that the diagnostic variable (FACS data) can be used to discriminate between two mutually exclusive states; in this case infectivity (as determined by PPE and cELISA  $>30\%$  in infected calves) or non-infectivity of test cultures following inoculation into calves. An ROC curve is a plot of sensitivity by  $(1 - \text{specificity})$  for each percent reduction value. The area under the ROC curve (AUC) is a common index used to summarize the information contained in the curve. In the case of a perfect test the AUC would be 1. The curve of the ROC plot can be used to determine the optimal sensitivity and specificity cut-off value to predict infectivity from flow cytometry data.

## Results

### Detection of parasitized erythrocytes

Differences in HE conversion were detected between uninfected and infected erythrocytes incubated with HE and analyzed by flow cytometry. Representative FACS histograms of HE treated samples are shown in Fig. 1. Cells in uninfected cultures did not convert HE as demonstrated by a single population of cells represented as a single histogram peak. Parasitized cells in infected cultures that converted HE to ethidium had an increase in

fluorescent intensity and appeared as a second population of cells, seen as a second peak, on the histogram.

### **Differences in mean percent reductions between antimicrobials**

The mean percent reduction in HE positive cells pooled across isolates for all antimicrobial dilutions are presented in Fig. 2. There was significant evidence of a difference in the mean percent reduction in HE positive erythrocytes between the enrofloxacin, imidocarb and oxytetracycline treated cultures on all days of the study (ANOVA  $P < 0.05$ ).

Comparisons for all pairs using Tukey-Kramer HSD indicated that after 24 h there were significantly fewer parasitized cells in the oxytetracycline ( $30.02 \pm 2.71\%$  reduction) and enrofloxacin ( $23.01 \pm 2.24\%$  reduction) cultures compared with the imidocarb ( $10.79 \pm 1.40\%$  reduction) ( $P < 0.01$ ). On day 2 and day 6 there were fewer parasitized erythrocytes in the enrofloxacin treated cultures and significantly more parasites in the oxytetracycline treated cultures. On the remaining days of the study there was no difference between enrofloxacin and imidocarb treated cultures but significantly more parasitized cells in the oxytetracycline treated cultures.

### **Differences in mean percent reductions between antimicrobial dilutions**

The mean percent reduction in HE positive cells for each antimicrobial dilution by isolate are presented in Fig. 3–5. The results for each antimicrobial tested are summarized below.

#### ***Enrofloxacin***

When data from both isolates were pooled, the percent reduction in HE positive cells increased with increasing antimicrobial concentrations in cultures exposed to enrofloxacin for 7 days. Cultures exposed to 4  $\mu\text{g/ml}$  enrofloxacin had a greater percent reduction in parasitized cells ( $44.72 \pm 2.43\%$ ) when compared with cultures exposed to 0.5–2  $\mu\text{g/ml}$  enrofloxacin ( $32.48 \pm 1.74\%$  to  $34.51 \pm 1.76\%$ ) ( $P < 0.01$ ). These cultures in turn had fewer HE positive cells than cultures exposed to 0.25  $\mu\text{g/ml}$  enrofloxacin ( $17.17 \pm 1.99\%$  reduction) ( $P < 0.01$ ).



In comparison with the other antimicrobials, cultures exposed to 4 µg/ml enrofloxacin had significantly fewer parasitized erythrocytes than cultures exposed to 0.25; 0.5 and 4 µg/ml imidocarb and 1–16 µg/ml oxytetracycline ( $P < 0.05$ ). Similarly, cultures exposed to 0.5–2 µg/ml enrofloxacin had significantly fewer parasitized erythrocytes than cultures exposed 0.25 µg/ml imidocarb and 1–16 µg/ml oxytetracycline ( $P < 0.05$ ).

### ***Imidocarb***

A paradoxical increase in HE positive cells was observed in cultures exposed to 4 µg/ml imidocarb for 7 days when data from both isolates was pooled. Cultures exposed to 1 and 2 µg/ml imidocarb ( $36.64 \pm 2.91\%$  and  $35.45 \pm 3\%$  reduction, respectively) had significantly greater percent reduction in parasitized cells when compared with cultures exposed to 0.5 and 4 µg/ml imidocarb ( $25.00 \pm 2.19\%$  and  $24.44 \pm 1.70\%$  reduction, respectively) ( $P < 0.05$ ). These cultures in turn had fewer HE positive cells than cultures exposed to 0.25 µg/ml imidocarb ( $14.77 \pm 1.71\%$ ) ( $P < 0.05$ ). In comparison with the other antimicrobials, cultures exposed to 1 and 2 µg/ml imidocarb had significantly fewer parasitized erythrocytes than cultures exposed to 0.25 µg/ml enrofloxacin ( $17.17 \pm 1.99\%$ ) and 1–16 µg/ml oxytetracycline ( $10.70 \pm 5.24$  to  $9.09 \pm 3.26\%$  reduction) ( $P < 0.05$ ).

### ***Oxytetracycline***

There was no relationship or significant difference between the mean percent reduction in parasitized erythrocytes and increasing antimicrobial dilutions in cultures exposed to oxytetracycline for 7 days ( $P > 0.05$ ) (Fig. 5). The greatest reduction in HE positive cells occurred within the first 24 h of exposure to oxytetracycline (Range:  $23.53 \pm 2.33\%$  (16 µg/ml OTC) to  $38.59 \pm 3.77\%$  (4 µg/ml OTC)). Over the 7 days of the study the mean percent reduction ranged from  $6.40 \pm 1.64\%$  to  $9.37 \pm 1.43\%$  in the cultures exposed to 8 µg/ml and 16 µg/ml oxytetracycline, respectively. In comparison with the other antimicrobials, cultures exposed to oxytetracycline had a significantly lower percent reduction in HE positive cells.

### **Differences between isolates**

There was considerable variation in the percent reduction in HE positive cells over the 7-day period of the study with statistical differences noted at various drug dilutions on several days. The mean percent reduction in HE positive cells for each isolate are presented in Fig. 3–5.

#### ***Enrofloxacin***

There was a significantly greater percent reduction in HE positive cells in the Virginia isolate cultures when compared with the Oklahoma cultures exposed to 0.5 µg/ml enrofloxacin ( $34.60 \pm 2.96\%$  compared to  $28.90 \pm 1.53\%$  ( $P = 0.04$ ) and 2 µg/ml enrofloxacin ( $40.31 \pm 2.36\%$  compared to  $28.72 \pm 1.96\%$  ( $P = 0.0005$ ) for 7 days. Numerically there was a greater percent reduction in HE positive cells in the Oklahoma isolate culture exposed to 4 µg/ml enrofloxacin ( $47.30 \pm 3.41\%$  compared to  $42.15 \pm 3.47\%$ ) although this was not statistically significant ( $P = 0.296$ ).

#### ***Imidocarb***

Significant differences between isolates were observed in cultures exposed to 0.5–4 µg/ml imidocarb. In all cases, a greater reduction in parasitized cells occurred in cultures infected with the Oklahoma isolate. This ranged from  $30.61 \pm 2.66\%$  compared to  $20.28 \pm 1.44\%$  reduction in the Virginia isolates exposed to 4.0 µg/ml imidocarb ( $P = 0.0018$ ) to  $48.93 \pm 3.97\%$  compared to  $24.34 \pm 1.95\%$  reduction in cultures exposed to 1.0 µg/ml imidocarb ( $P = 0.0001$ ).

#### ***Oxytetracycline***

There was a significantly greater percent reduction in HE positive cells in the Virginia isolate cultures when compared with the Oklahoma cultures exposed to 8 µg/ml oxytetracycline:  $11.43 \pm 2.83\%$  compared to  $1.35 \pm 0.68\%$ , respectively ( $P = 0.002$ ). The difference between isolates exposed to 16 µg/ml oxytetracycline was approaching significance,  $12.06 \pm 1.94\%$  (VGN) compared to  $6.67 \pm 1.98\%$  (OK) ( $P = 0.059$ ) for 7 days.

### Testing infectivity of selected cultures

Percent reduction in HE positive cells obtained by FACS was used to select pairs of cultures infected with the Virginia and Oklahoma isolates treated with 0.25 and 4.0 µg/ml enrofloxacin, 0.25 and 1.0 µg/ml imidocarb and 1.0 and 16.0 µg/ml oxytetracycline for inoculation into calves. These data and the cELISA results used to monitor seroconversion in calves following inoculation is summarized in Table 1 and Fig. 6.

Cultures containing uninfected blood and those infected with the Oklahoma isolate exposed to 4.0 µg/ml enrofloxacin and the Virginia and Oklahoma isolates exposed to 1.0 µg/ml imidocarb failed to elicit a serological response or parasitemia in calves (cELISA <30% inhibition). Following splenectomy these animals failed to demonstrate evidence of infection. The remaining calves developed a parasitemia and seroconverted within 10 to 31 days post-inoculation. Linear regression of cELISA against days post-inoculation indicated that calves with higher percent reductions tended to have smaller intercepts and/or reduced slopes and took longer to seroconvert.

### Comparison between FACS and *in vivo* infectivity

The results of the ROC analysis are summarized in Table 2. The area under the ROC curve ranged from 0.67 on day 1 to 0.95 on day 3. On day 1, the ability of FACS to detect infection in culture (sensitivity) was 47% and ability of FACS to detect sterilization of culture (specificity) was 91% at a cut-off value of 7.89% HE positive cells. This increased to a sensitivity of 88% and a specificity of 86% at a cut-off of 14% HE positive cells on day 3.

### Discussion and conclusions

The purpose of the present study was to utilize whole blood culture and flow cytometric analysis (FACS) to evaluate the effect of enrofloxacin, imidocarb and oxytetracycline against an Oklahoma and Virginia isolate of *A. marginale*. The percent reduction in HE positive cells was used to select cultures to inoculate into calves in order to determine the infectivity of the culture. These data indicate that the MRC of enrofloxacin against the Oklahoma isolate of *A. marginale* was greater than 0.25 µg/ml but ≤4.0 µg/ml. However, the MRC of enrofloxacin against the Virginia isolate was greater than 4.0 µg/ml.

In contrast the MRC of imidocarb against both isolates was between 0.25 µg/ml and 1.0 µg/ml. The MRC of oxytetracycline required to be effective against anaplasmosis is greater than 16.0 µg/ml.

The data presented suggest that flow cytometry can be a reliable predictor of infectivity although this varied between days of the study. The area under the ROC curve ranged from 0.67 on day 1 to 0.95 on day 3. ROC analysis has been increasingly used for the evaluation of clinical laboratory tests, however, its use in medical and veterinary literature is limited (Greiner et al., 2000). The area under the ROC curve is a global summary statistic of diagnostic accuracy. According to an arbitrary guideline suggested by Swets (1988), these data can distinguish between non-informative ( $AUC = 0.5$ ), less accurate ( $0.5 < AUC \leq 0.7$ ), moderately accurate ( $0.7 < AUC \leq 0.9$ ), highly accurate ( $0.9 < AUC < 1.0$ ) and perfect tests ( $AUC = 1$ ). Based on these guidelines the optimum correlation between FACS data and infectivity occurred on day 3 when the AUC was 0.95. This would suggest that future susceptibility studies using this method may be conducted over 3 as opposed to 7 days as in the present study.

Our results indicate that enrofloxacin inhibits *A. marginale* in a concentration dependent manner. Enrofloxacin is a fluoroquinolone antimicrobial that inhibits bacterial DNA-gyrase (Topoisomerase II) and Topoisomerase IV (Blondeau, 2004). This activity prevents DNA supercoiling and decatenation of original chromosomes and replicates. Two published reports indicate that enrofloxacin (Baytril®, Bayer Animal Health) is effective against acute *A. marginale* infections *in vivo* at dose rates of 5–10 mg/kg (Schroder et al., 1991; Guglielmone et al., 1996). To our knowledge this is the first report attempting to elucidate a minimum inhibitory concentration of enrofloxacin against *A. marginale*. These data, in conjunction with published pharmacokinetic and pharmacodynamic information, may facilitate an assessment of the potential use of enrofloxacin against persistent *A. marginale* infections.

The bactericidal activity of fluoroquinolone antimicrobials is dependant on the ratios of the area under the plasma drug concentration curve (AUC) to the minimum inhibitory concentration (MIC) for bacteria. Studies reviewed by Drusano et al. (2001) investigating this relationship in human *Pseudomonas aeruginosa* infections found that the optimal

AUC/MIC ratio for a fluoroquinolone was  $\geq 125$ . The AUC from 0–12 h following subcutaneous administration of enrofloxacin to calves challenged with *Mannheimia haemolytica* at 8 mg/kg to calves was 7.51 mg·h/L (Terhune et al., 2005). Kaartinen et al. (1997) reported an AUC of 13.94 mg·h/L in one-day-old calves and 6.73 mg·h/L in one-week-old calves. If the AUC/MIC ratio described in bacteria applies to *A. marginale*, this would suggest that a prohibitive increase in drug dose would be required in order to achieve rickettsiacidal plasma concentrations *in vivo*.

It should be noted that enrofloxacin is de-ethylated to ciprofloxacin in calves. Ciprofloxacin contributes between 10% and 27% of the total concentration in serum (Kaartinen et al., 1997). The study reported herein only investigated the effect of enrofloxacin and not ciprofloxacin *in vitro*. Antimicrobial susceptibility studies involving ciprofloxacin have been conducted against *Anaplasma phagocytophilum* which causes human granulocytic anaplasmosis (HGE). When this organism is grown in the human promyelocytic cell line, the MIC of ciprofloxacin is between 1 µg/ml and 2 µg/ml (Klein et al., 1997; Branger et al., 2004). Further studies to evaluate the MIC of fluoroquinolones against *A. marginale* are necessary to fully assess the potential for eliminating carrier infections.

The present study examined two geographically and phylogenetically distinct isolates of *A. marginale* as described by de la Fuente et al. (2001). Our study revealed statistical difference between the two isolates exposed to 0.5 and 2 µg/ml enrofloxacin for 7 days. However only the Oklahoma isolate exposed to 4.0 µg/ml failed to infect a healthy calf. Maurin et al. (2003) demonstrated differences in the MIC of the fluoroquinolone levofloxacin against different geographic isolates of *Anaplasma phagocytophilum*. *In vitro* MICs varied from 0.06–0.5 µg/ml which is reported to be close to the maximum levels achievable in human serum. These researchers previously demonstrated a DNA gyrase-mediated natural resistance to fluoroquinolones in *Ehrlichia chaffeensis* and *Ehrlichia canis* (Maurin et al., 2001). This corresponds to a single amino acid difference in *gyrA* which encodes the A subunit of DNA gyrase in *A. phagocytophilum*. Our study suggested that susceptibility differences may exist between the Oklahoma and Virginia isolate of *A. marginale*, but the exact mechanism still remains to be elucidated. This finding would limit

the widespread use of enrofloxacin in field cases of *A. marginale*. Federal (USA) law also prohibits the extra-label use of enrofloxacin in food producing animals.

In this study, imidocarb demonstrated paradoxically more HE positive cells in cultures exposed to 4 µg/ml than cultures exposed to 1 and 2 µg/ml. A statistical difference was also found between the two isolates exposed to 0.5–4 µg/ml imidocarb for 7 days.

Imidocarb is a carbanilide derivative with antiprotozoal activity. It is usually administered as the dipropionate salt by subcutaneous or intramuscular injection to cattle at a dose rate of 2.1 mg/kg. The mode of action of action of imidocarb is uncertain though two mechanisms have been proposed: interference with the production and/or utilization of polyamines, or prevention of entry of inositol into the erythrocyte containing the parasite (EMA, 2001).

Imidocarb dipropionate has been used for over 30 years in the treatment of bovine anaplasmosis in certain territories (McHardy and Simpson, 1974). Roby and Mazzola (1972) found that two injections of imidocarb, administered at 5 mg/kg 14 days apart, eliminated *A. marginale* from carrier animals. Pharmacokinetic data and reports on plasma concentrations of imidocarb that may be effective for therapy in cattle are lacking. Studies in goats, dogs and horses suggest that this compound has a large volume of distribution resulting in a prolonged elimination half-time (Abdullah and Baggot, 1983; Belloli et al., 2002). These observations are confirmed by studies demonstrating a prolonged retention of the drug in edible tissues (EMA, 2001). This has restricted the use of this compound in food producing animals in many territories.

The paradoxical effect of antibiotics is defined as a substantially reduced bacterial killing at antibiotic levels above the minimum bactericidal concentration *in vitro* (Holm et al., 1991). This phenomenon was originally described for  $\beta$ -lactam antibiotics against Gram positive bacteria. Subsequent studies have described this phenomenon with aminoglycosides against Gram negatives and pefloxacin against *Escherichia coli* (Lorian et al., 1979; Yourassowsky, 1986). The clinical significance of the paradoxical effect is unknown. However, based on pharmacokinetic data derived from other species, it would appear unlikely that imidocarb concentrations greater than 1 µg/ml are attainable in bovine plasma

without significant toxic effects. This would suggest that the paradoxical effect observed *in vitro* may have little clinical relevance.

Oxytetracycline treatment was less efficacious at reducing HE positive cells than treatment with enrofloxacin or imidocarb. There was no relationship between drug concentration and percent reduction in HE positive cells in these cultures. The greatest reduction occurred within the first 24 h of exposure to oxytetracycline. A statistical difference between the two isolates exposed to 8 µg/ml oxytetracycline was detected, with the Virginia isolate appearing more susceptible.

Oxytetracycline is a tetracycline derivative obtained from *Streptomyces rimosus*. Tetracyclines bind to ribosomes and mRNA. Inhibition of protein synthesis is mediated principally through reversible binding with the 30S ribosomal subunit. In bacteria, this binding blocks the attachment of aminoacyl-tRNA to the A site of the mRNA-ribosome complex (Scholar and Pratt, 2000). Tetracycline antimicrobials are bacteriostatic rather than bacteriocidal and the activity of these compounds is believed to be dependant on the time that drug concentrations remain above the minimum inhibitory concentration (MIC) for the target organism. Our data suggest that oxytetracycline is rickettsiastatic and that a competent host immune response is necessary for elimination of the pathogen.

Blouin et al. (2002) used cultivated *A. marginale* in a cell line derived from embryos of *Ixodes scapularis* ticks to examine the effect of tetracycline on the organism. Tetracycline doses of 5, 10, 20 and 100 µg/ml resulted in significant inhibition of *A. marginale* growth as determined by ELISA. Infected cell cultures treated with medium containing 20 µg/ml tetracycline proved non-infective when inoculated into susceptible splenectomized calves. These data suggest that the MRC may be somewhere between 16–20 µg/ml tetracycline although this does not account for possible differences related to *in vitro* culture techniques.

*In vivo* pharmacokinetic studies have found that the maximum plasma concentration ( $C_{max}$ ) following IM administration of a 200mg/ml long-acting oxytetracycline formulation at 20mg/kg was 8.56 µg/ml achieved at 2.53 h after injection. The  $C_{max}$  of a new formulation of oxytetracycline (Tetradure LA-300®, Merial LTD) containing 300mg/ml was 10.72 µg/ml achieved at 4.64 h after administration (Clark and Dowling, 2003). These results suggest that plasma concentrations of these formulations of oxytetracycline following parenteral

administration are insufficient to kill *A. marginale* based on the MIC determined in the present study.

In previous studies in which successful clearance of persistent *A. marginale* infections was achieved, oxytetracycline was administered intravenously to cattle at 11–22 mg/kg for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Intramuscular oxytetracycline administered at 20mg/kg on 2, 3 or 4 occasions at intervals ranging from 3–7 days was also reported to be effective at eliminating carrier infections (Roby et al., 1978; Kuttler et al., 1980; Magonigle and Newby, 1982; Kuttler, 1983; Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem et al., 1988). A recent study conducted by our research group demonstrated that the current recommended OIE treatment protocol of 5 injections of oxytetracycline at 22 mg/kg intravenously did not eliminate persistent Oklahoma isolate infections (Coetzee et al., 2005). These conflicting reports suggest that possible differences in susceptibility between isolates may exist. This hypothesis is supported by the recent identification of two multidrug resistance pumps in the genome of *A. marginale* (Brayton et al., 2005). However, the clinical significance or activity of these pumps has yet to be elucidated.

The results of the present study demonstrate that short term blood culture and FACS can be used to determine the antimicrobial susceptibility of *A. marginale* and to evaluate the efficacy of novel antimicrobials *in vitro*. Cultures infected with the Oklahoma isolate exposed to 4.0 µg/ml enrofloxacin and the Virginia and Oklahoma isolate exposed to 1.0 µg/ml imidocarb for 7 days were sterilized. Enrofloxacin inhibited *A. marginale* in a concentration-dependent manner whereas higher concentrations of imidocarb were paradoxically less effective at reducing the number of parasitized erythrocytes. Oxytetracycline was the least efficacious antimicrobial tested and there was no relationship between oxytetracycline concentration and percent reduction in HE positive cells. With the exception of cultures exposed to enrofloxacin at 4.0 µg/ml, the isolate differences observed *in vitro* did not translate to differences in infectivity in this experiment. Further studies are required to fully elucidate the susceptibility profile of different *A. marginale* isolates. These data are essential to facilitate the development of successful chemotherapeutic protocols for the elimination of persistent *A. marginale* infections.



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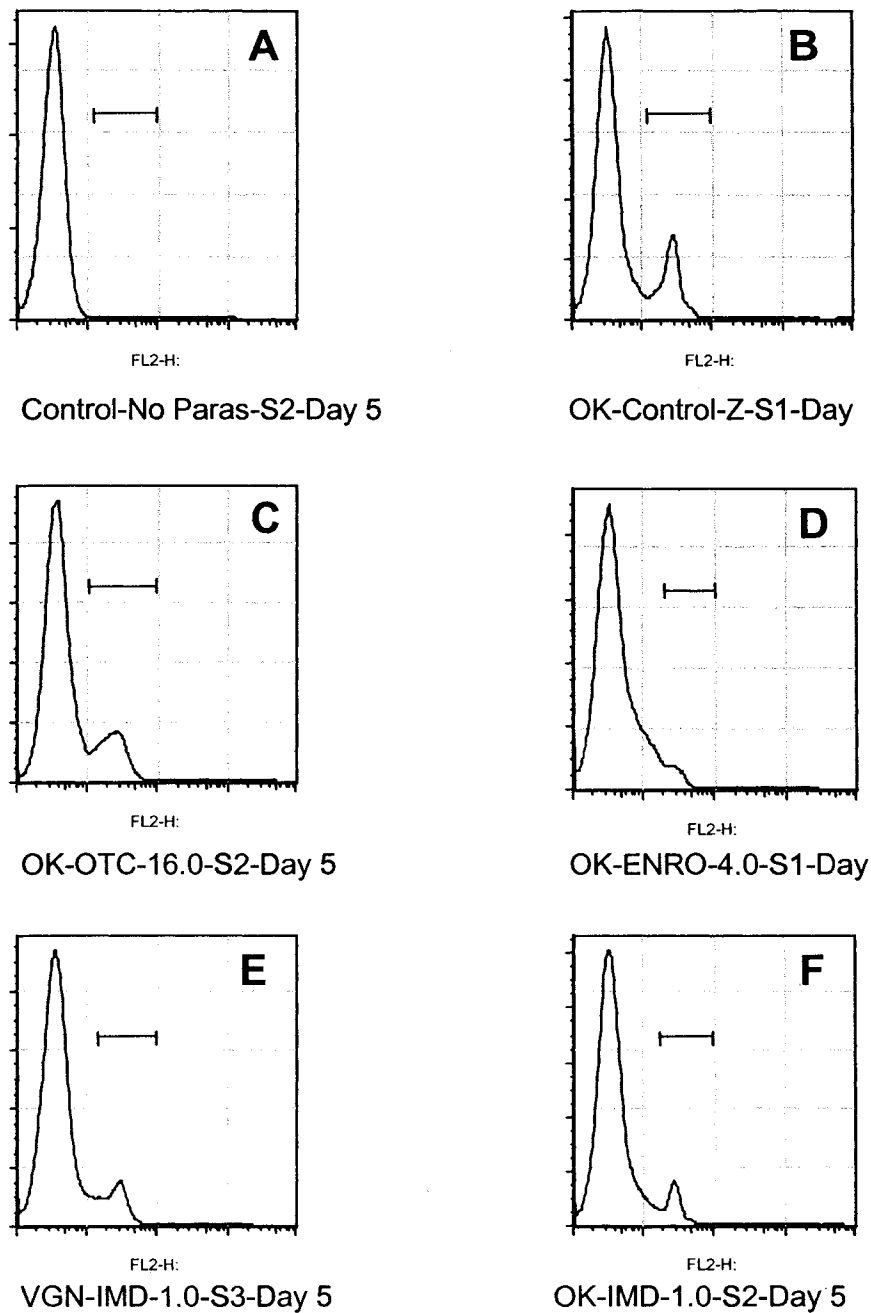
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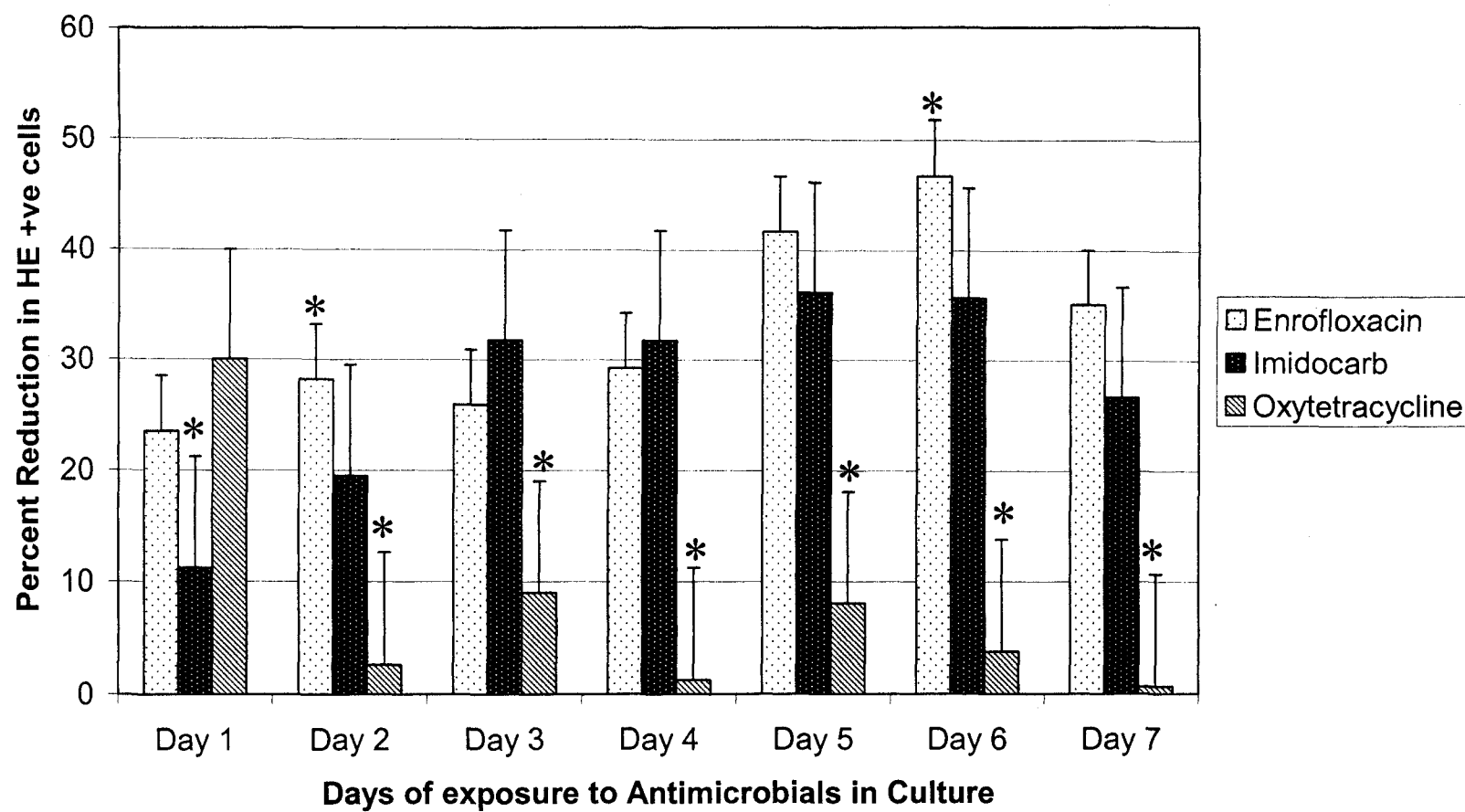
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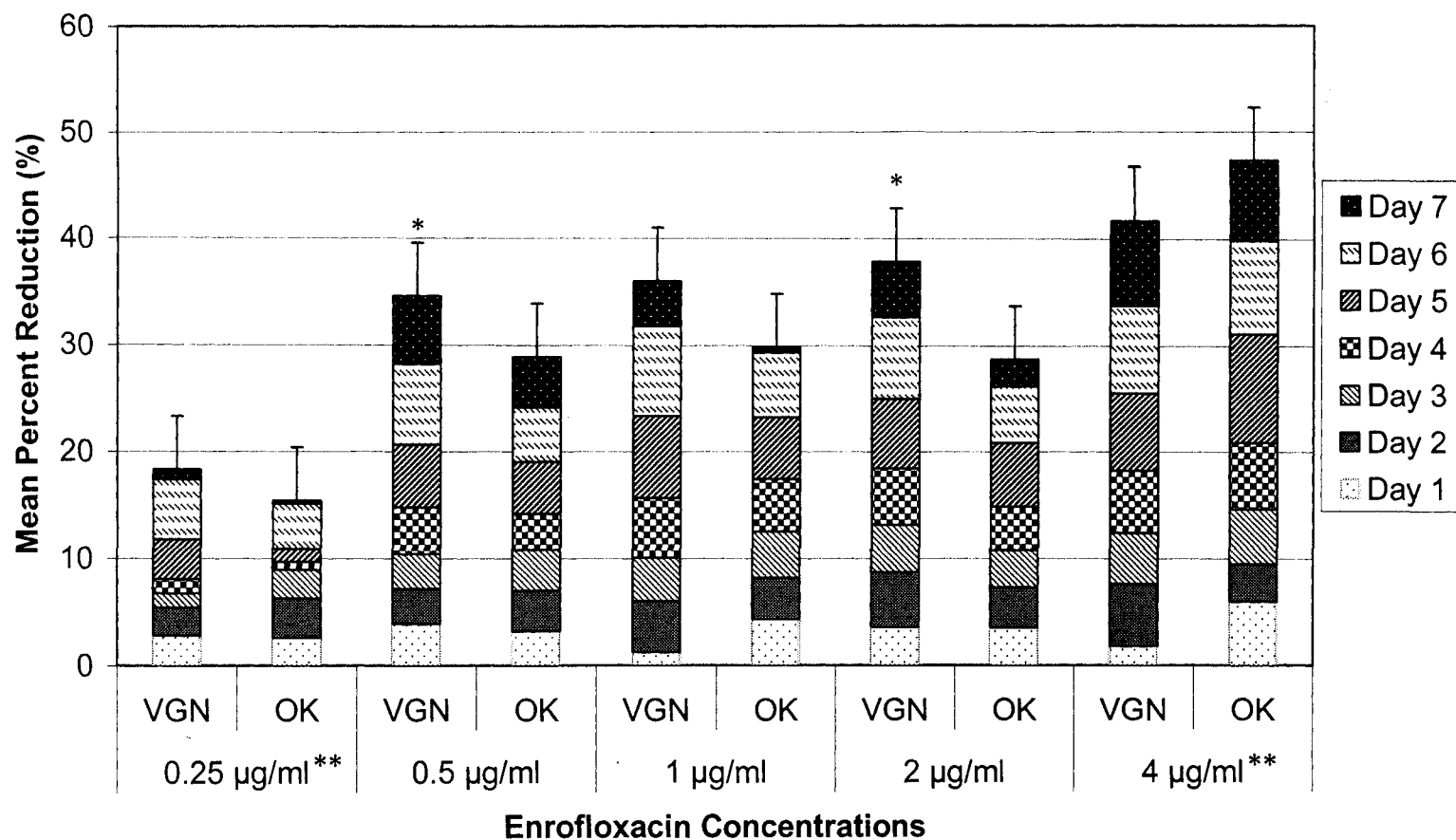


**Fig. 1: Flow Cytometry Histograms.** Cells in uninfected cultures did not convert Hydroethidine (HE) as demonstrated by a single histogram peak population (A). Parasitized cells infected cultures converted HE to ethidium bromide and appeared as a second gated (18.4%) population in untreated cultures (B). Selected Oklahoma (OK) and Virginia (VGN) isolate cultures exposed to 16 $\mu$ g/ml oxytetracycline (OTC) (C), 4  $\mu$ g/ml enrofloxacin (ENRO) (D), and 1  $\mu$ g/ml imidocarb (IMD) (E) and (F) for 5 days are presented. Samples D-F failed to infect calves after 7 days of culture.



**Figure 2: Comparison between the total mean percent reduction in HE positive cells in cultures treated with enrofloxacin, imidocarb and scytetracycline for 7 days**

\*Significant difference in mean % reduction between antimicrobials ( $p < 0.05$ )

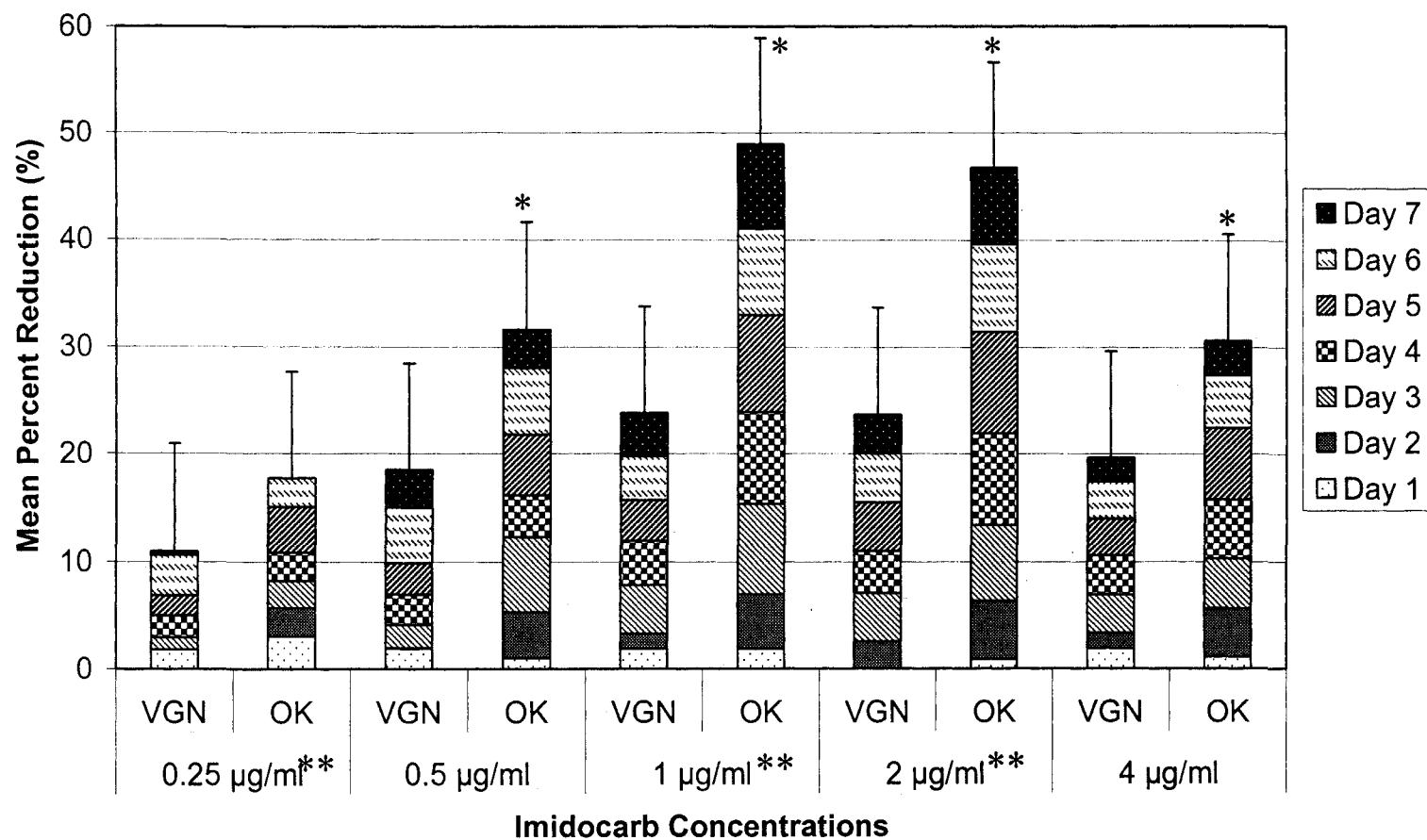


**Figure 3: Comparison between the mean percent reduction in hydroethidine positive cells in the Virginia (VGN) and Oklahoma (OK) isolates following exposure to enrofloxacin for 7 days**

\*Significant difference in mean % reduction between isolates ( $p < 0.05$ )

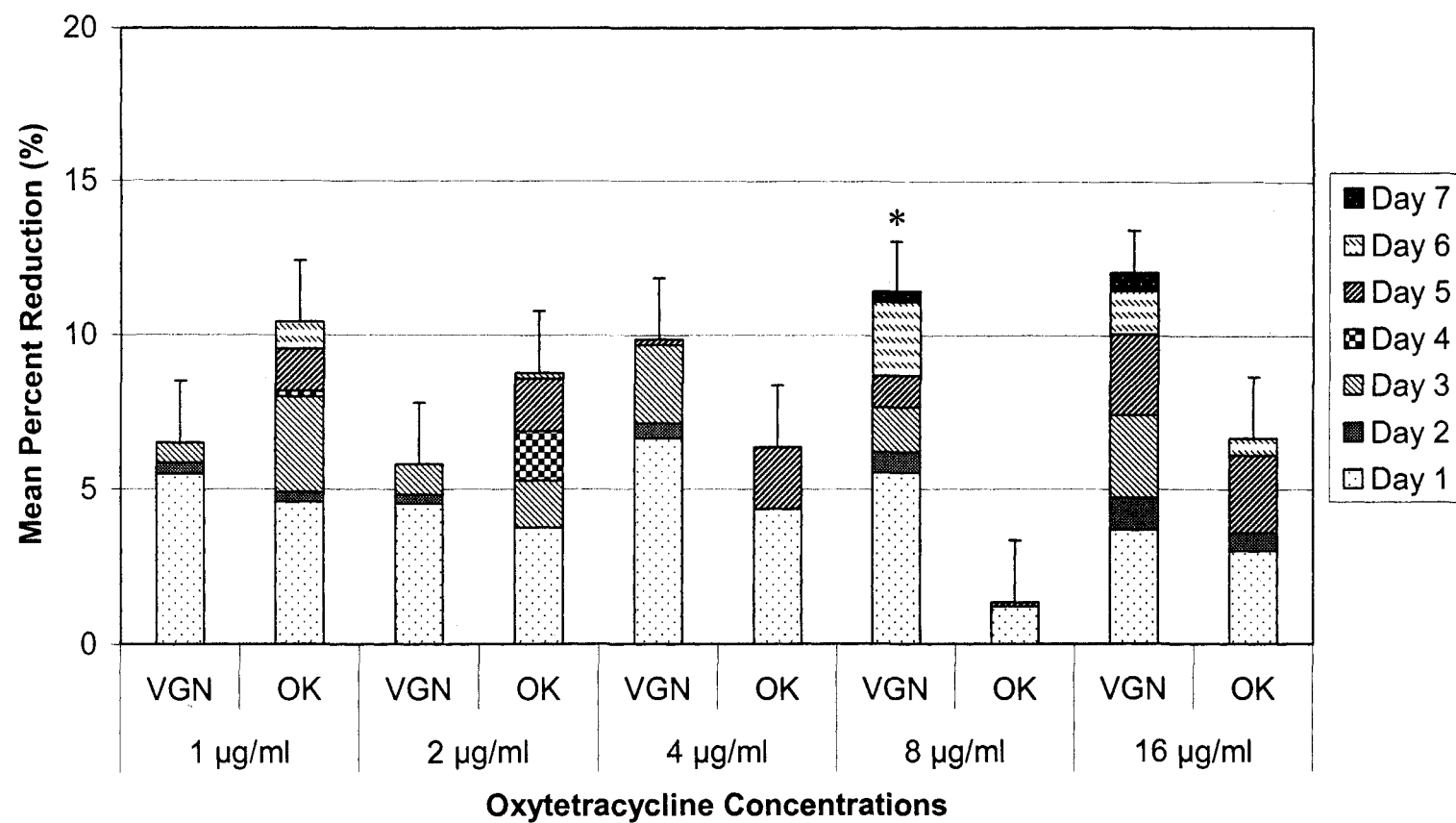
\*\*Significant difference in mean % reduction between antimicrobial dilutions pooled across isolates ( $p < 0.01$ )





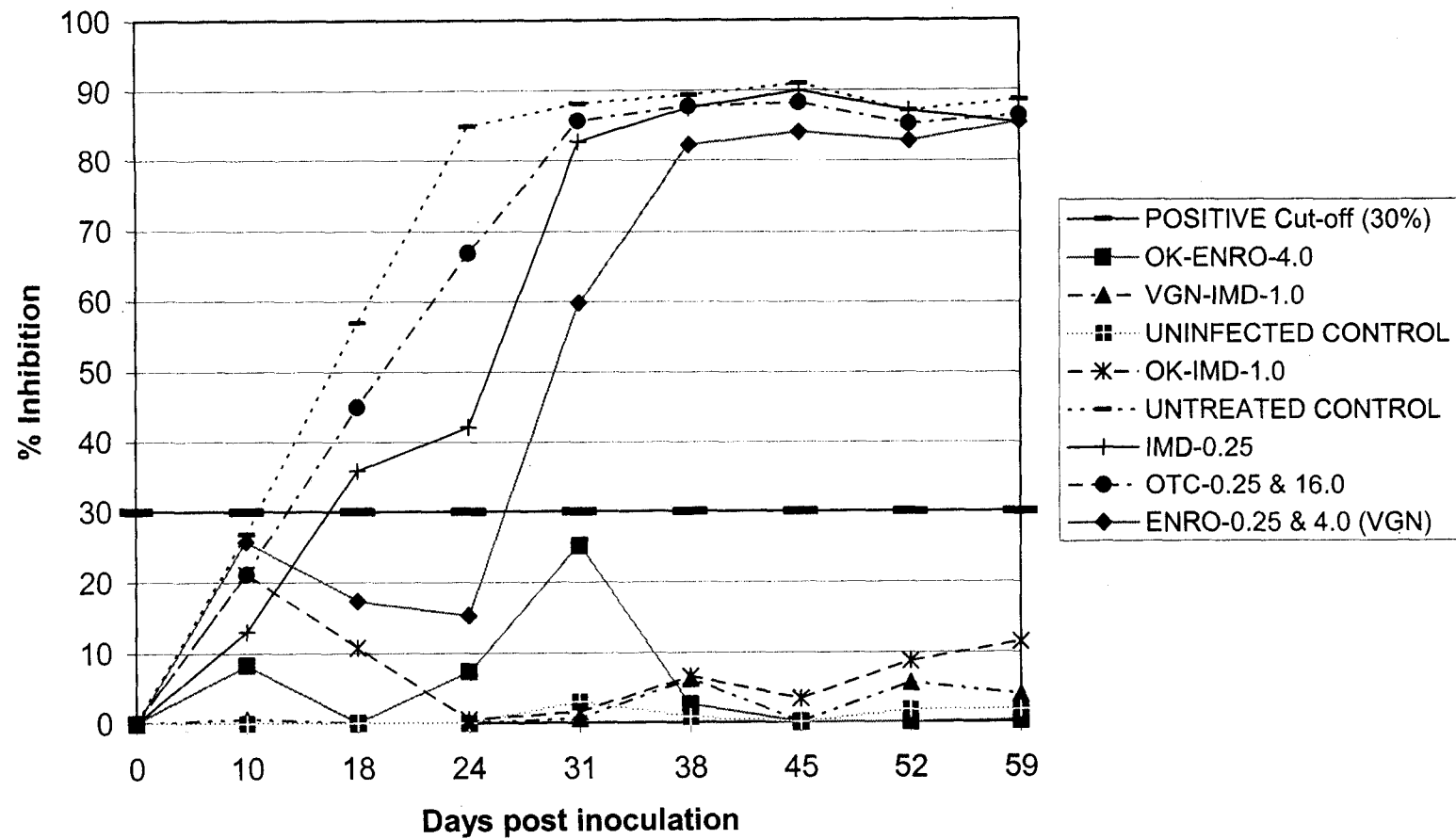
\*\*Significant difference in mean % reduction between antimicrobial dilutions across isolates ( $p < 0.01$ )

\*Significant difference in mean % reduction between isolates ( $p < 0.05$ )



**Figure 5. Comparison between the mean percent reduction in hydroethidine positive cells in the Virginia (VGN) and Oklahoma (OK) isolates following exposure to oxytetracycline for 7 days**

\*Significant difference in mean % reduction between isolates ( $p < 0.05$ )



**Figure 6: Serological responses measured by competitive ELISA (cELISA) following inoculation of selected erythrocyte cultures into Holstein calves**

**Table 1: Flow cytometry data used for selecting erythrocyte cultures and competitive ELISA data collected from calves following inoculation of cultures incubated with or without the addition of antimicrobials for 7 days**

Isolate <sup>a</sup> -Antimicrobial <sup>b</sup> -Dilution <sup>c</sup>	Mean % Reduction by FACS following 7 days of culture	cELISA (% inhibition)			cELISA Linear Regression		
		Pre-inoculation	59 Days Post-inoculation	Days to Seroconversion (cELISA > 30%)	Intercept	Slope	p-Value
Uninfected Control	n/a	0	1.76	n/a	-0.12	0.03	0.22
OK-Infected Control	n/a	0	87.83	10 days	23.35	1.43	0.003
VGN- Infected Control	n/a	0	89.43	18 days	24.18	1.45	0.006
OK-ENRO- 0.25 µg/ml	15.38%	0	82.50	31 days	-3.51	1.94	0.013
VGN-ENRO- 0.25 µg/ml	18.34%	0	86.84	24 days	4.32	1.63	0.0001
OK-ENRO- 4.0 µg/ml	47.30%	0	0.17	n/a	6.75	-0.06	0.71
VGN-ENRO- 4.0 µg/ml	42.15%	0	86.94	31 days	-8.88	1.75	0.0007
OK-IMD- 0.25 µg/ml	17.04%	0	88.93	18 days	18.41	1.55	0.002
VGN-IMD-0.25 µg/ml	11.56%	0	81.65	31 days	-5.13	1.79	0.002
OK-IMD-1.0 µg/ml	48.93%	0	11.44	n/a	6.89	0.01	0.96
VGN-IMD-1.0 µg/ml	24.34%	0	3.96	n/a	-0.73	0.085	0.062
OK-OTC-1.0 µg/ml	15.03%	0	89.43	24 days	7.69	1.68	0.0004
VGN-OTC-1.0 µg/ml	6.38%	0	86.54	18 days	25.40	1.38	0.0047
OK-OTC-16.0 µg/ml	6.12%	0	82.85	18 days	17.45	1.47	0.009
VGN-OTC-16.0 µg/ml	12.06%	0	86.84	10 days	14.41	1.54	0.0003

<sup>a</sup>Isolate: VGN-Virginia isolate; OK-Oklahoma isolate

<sup>b</sup>Antimicrobial: ENRO-enrofloxacin; IMD-imidocarb; OTC-oxytetracycline

<sup>c</sup>Dilution: 0.25 µg/ml; 1.0 µg/ml; 4.0 µg/ml; 16.0 µg/ml

**Table 2: Summary of the receiver-operating characteristic (ROC) analysis used to determine the percent HE positive cells which most closely correlated with sterilization of anaplasmosis infections in culture on each day of the study.**

Study Day	Area Under ROC Curve (AUC <sup>a</sup> )	Cut-off Value (HE + ve cells)	Sensitivity	Specificity
1	0.67	7.89%	47%	91%
2	0.78	16.20%	60%	95%
3	0.95	14%	88%	86%
4	0.89	11.70%	80%	100%
5	0.86	12.10%	73%	100%
6	0.79	7.69%	68%	100%
7	0.84	6.15%	77%	100%

<sup>a</sup>AUC: Non-informative, AUC=0.5; less accurate,  $0.5 < \text{AUC} \leq 0.7$ ; moderately accurate,  $0.7 < \text{AUC} \leq 0.9$ ; highly accurate,  $0.9 < \text{AUC} < 1.0$ ; and perfect tests, AUC = 1 (Swets, 1988).

## CHAPTER 5. INHIBITORY EFFECT OF ENROFLOXACIN AGAINST SEVERE EXPERIMENTAL *ANAPLASMA MARGINALE* INFECTIONS IN SPLENECTOMIZED CALVES

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### Abstract

*Anaplasma marginale* is the most prevalent tick-borne pathogen of cattle world wide. Oxytetracycline and imidocarb dipropionate are currently the only compounds available to treat acute anaplasmosis infections. This study tested the efficacy of enrofloxacin (Baytril®100, Bayer Animal Health) against severe experimental *A. marginale* infections in splenectomized calves. Six Holstein steers, aged less than 8 months, were confirmed anaplasmosis free by competitive ELISA (cELISA) and then splenectomized. At least 6 weeks after splenectomy, two calves were inoculated with a West Coast (St. Maries) isolate of *A. marginale*; three calves were infected with an Oklahoma isolate while the remaining calf was infected with a Virginia isolate. Calves developed peak rickettsemias ranging from 28.6–55% parasitized erythrocytes (PPE) within 15–39 days after infection. The packed cell volume (PCV) at this time ranged from 12–32.5%. Two animals infected with the Oklahoma isolate were designated untreated control animals. The remaining four animals received two subcutaneous injections of enrofloxacin at a dose rate of 12.5 mg/kg, administered 48 h apart. Following treatment, PPE and PCV measurements were collected for approximately 6 weeks. These data were analyzed using analysis of variance (ANOVA), paired student t-tests and a simple linear regression model. Both untreated control calves became moribund when the PCV dropped below 10% and were euthanized for humane reasons. The difference in PPE between the treated and control animals was approaching significance at this time point ( $P = 0.061$ ). A precipitous decline in PPE from a pre-treatment mean of 39.13% to less than 1% was observed in the first 12 days following treatment with enrofloxacin ( $P < 0.0002$ ). A recrudescence of *A. marginale* parasites was observed in all treated animals within 30 days

following treatment. In three cases the subsequent wave of rickettsemia was less severe and self limiting. However one animal contracted respiratory disease and had to be euthanized. These data indicate that enrofloxacin administered at 12.5 mg/kg twice, 48 h apart ameliorates, but does not clear, *A. marginale* infection in splenectomized calves. Untreated animals subjected to this model died. Further studies are warranted to investigate whether a dose regimen of enrofloxacin can be identified to eliminate *A. marginale* infection from persistently infected cattle.

## Introduction

Anaplasmosis, caused by the rickettsial hemoparasite, *Anaplasma marginale*, is the most prevalent tick-transmitted disease of cattle worldwide (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2003). Prior to the development of imidocarb dipropionate and the tetracycline antimicrobials, a variety of chemotherapeutic agents, including arsenicals, antimalarials, antimony derivatives and dyes, were used to treat acute anaplasmosis. These compounds had little if any chemotherapeutic effect (Potgieter and Stoltz, 1994).

Chlortetracycline and oxytetracycline are the only compounds available for prevention and control of acute anaplasmosis in the United States. *Anaplasma* infections are not sterilized at the usual recommended therapeutic doses of the tetracycline drugs (Kuttler and Simpson, 1978; Stewart et al., 1979). There are currently no antimicrobials labeled for the elimination of persistent infections in carrier animals. In previous studies in which successful clearance of persistent *A. marginale* infections was achieved, oxytetracycline was administered intravenously to cattle at 11–22 mg/kg for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Intramuscular oxytetracycline administered at 20 mg/kg on two, three, or four occasions at intervals ranging from 3–7 days was also reported to be effective at eliminating carrier infections (Roby et al., 1978; Kuttler et al., 1980; Magonigle and Newby, 1982; Kuttler, 1983; Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem et al., 1988).

Splenectomized calves have been used as a model for evaluating the efficacy of antimicrobials against *A. marginale* (Miller et al., 1952; Roby, 1972; Stewart et al., 1979; Kuttler, 1986). Successful clearance of anaplasmosis in splenectomized calves has not been

achieved with either imidocarb or oxytetracycline (Stewart et al., 1979; Kuttler, 1986). This may be due to inadequate serum drug concentrations achieved with these products or the absence of an adequate immune response to work in combination with the antimicrobial to bring about elimination of the parasite (Kuttler et al., 1980).

Enrofloxacin is a fluoroquinolone antimicrobial that inhibits bacterial DNA-gyrase (Topoisomerase II) and Topoisomerase IV (Blondeau, 2004). This activity prevents DNA supercoiling and decatenation of original chromosomes and replicates. The bactericidal activity of enrofloxacin is concentration dependent, with susceptible bacteria cell death occurring within 20–30 min of exposure. Two published reports indicate that enrofloxacin (Baytril®, Bayer Animal Health) is effective against acute *A. marginale* infections at dose rates of 5–10 mg/kg (Schröder et al., 1991; Guglielmone et al., 1996). However the dynamics of the infection following treatment and the potential for elimination of the carrier state was not assessed. The need for additional trials to study whether enrofloxacin is useful to control severe anaplasmosis infections was also identified (Guglielmone et al., 1996).

The purpose of this study was to test the efficacy of enrofloxacin (Baytril®100, Bayer Animal Health) against experimental *A. marginale* infection in splenectomized calves. The specific aims were to evaluate the efficacy of enrofloxacin administered subcutaneously to severely infected animals (>25% parasitized erythrocyte (PPE) twice, 48 h apart at a dose rate of 12.5 mg/kg. In addition it was our intent to study the dynamics of *A. marginale* infection following treatment in order to ascertain whether this antimicrobial could be efficacious against persistent *A. marginale* infections caused by a variety of isolates.

## **Materials and methods**

This protocol was approved by the Committee on Animal Care (COAC) at Iowa State University.

### **Experimental cattle**

Five Holstein calves were obtained from the Iowa State University Dairy Breeding Research facility at Ankeny, IA and one animal was purchased from northwest Iowa. Both herds had no recorded cases of anaplasmosis. The individual animal details are summarized



in Table 1. Calves were aged approximately 150–240 days and weighed between 68 and 169 kg at the time of treatment. Animals were confirmed free of *A. marginale* antibodies by competitive enzyme linked immunoabsorbent assay (Anaplasma Antibody Test Kit, VMRD Inc., Pullman, WA) (cELISA) (Torioni De Echaide et al., 1998). Splenectomies were performed when calves were approximately 3 months old using the technique described by Thompson et al. (1992).

### **Housing and husbandry**

Calves were housed in a Biolevel 2 livestock infectious disease isolation facility at Iowa State University. These were housed in pairs and fed approximately 3 lbs of a corn-based ration twice a day. The ration was manufactured without the addition of any oxytetracycline antibiotics. Animals also received a small amount of grass hay twice a day and water *ad libitum* for the duration of the study.

### **Experimental infection with anaplasmosis**

Approximately 6–8 weeks after splenectomy, two calves were infected with 20 ml of blood from a carrier animal experimentally infected with a West Coast (St. Maries) isolate of *A. marginale*. Two control calves were similarly infected with blood infected with an Oklahoma isolate of anaplasmosis. Of the two remaining calves, one was infected with 10 ml of blood stabilate containing an Oklahoma isolate with 31.6% parasitized erythrocytes. The sixth calf received 10 ml of blood stabilate containing a Virginia isolate with 35.5% parasitized erythrocytes. The stabilates were prepared from packed erythrocytes washed in phosphate buffered saline (PBS) and frozen in liquid nitrogen at a 1:1 ratio with PBS containing 10% dimethylsulphoxide (DMSO). Stabilates were maintained frozen in liquid nitrogen and dry ice prior to inoculation.

### **Post-infection monitoring**

Following infection, the calves were monitored daily for the clinical signs of anaplasmosis, including anorexia, depression and listlessness. Blood samples were collected at least once a week post-infection for determination of PPE, PCV and cELISA serology. Blood samples were collected by jugular venipuncture using 18 G, 1 in. needles (Air-tite

Products Co., Inc., VA). For serum collection, 10 ml Monoject® No Additive Sterile glass tubes (Sherwood Medical, St Louis, MO) were used. For whole blood (PCV and PPE), 7 ml K<sub>3</sub>EDTA glass tubes (Beckton Dickenson Vacutainer Systems, NJ) were used. Blood in EDTA was refrigerated prior to PCV testing or packaged in insulated material for overnight delivery by courier to Oklahoma State University for determination of the PPE.

Blood smears for PPE determination were stained using a 30-s, three-step staining technique (Hema 3® Staining System, Fisher Scientific) comparable to the Wright-Giemsa method. Two slides were prepared for each blood sample and examined for the presence of *A. marginale* at 100x magnification using a grid. A total of 500 cells were counted within the 4 squares of the grid and the number of infected cells was recorded. The PPE was the number of infected cells divided by the total number of cells counted, multiplied by 100.

Serologic testing by cELISA was conducted by the Iowa State University Veterinary Diagnostic Laboratory. The test was conducted in accordance with the method described in the OIE Manual of Standards for diagnostic tests and vaccines and the manufacturer recommendations (OIE, 2000; VMRD, 2003). Results are given as percentage inhibition. PCVs were determined by partially filling heparinized capillary tubes (Chase Scientific Glass Inc., Rockwood, TN) with blood, which were then centrifuged for 3 min using an Adams Micro-Hematocrit centrifuge (Model CT 2900, Clay Adams, Inc., New York).

### **Treatment**

Treatment with enrofloxacin (Baytril®100, Bayer Animal Health, Shawnee Mission, KS) was initiated 19–25 days after experimental infection when the PPE ranged from 28.6%–53.2%. Each animal received two subcutaneous injections of enrofloxacin at a dose rate of 12.5 mg/kg, administered 48 h apart. Federal (USA) law prohibits the extra-label use of enrofloxacin in food-producing animals. Accordingly, experimental animals were kept in an isolation facility after treatment and were euthanized and incinerated at the end of the study.

### **Post-treatment monitoring**

Blood samples were collected at least once a week for approximately 42 days post-treatment for PPE, PCV and cELISA serology.

## Statistics

Data were entered into a spreadsheet program (Excel 2003, Microsoft Corporation, Redmond, WA) for subsequent calculation and manipulation. The mean  $\pm$  SEM (standard error of the mean) were calculated for all parameters. Hypothesis tests were conducted using JMP 5.1.2 analytical software (SAS Institute, INC, Cary, NC). Group differences between treated and control animals were analyzed using a Student t test. Statistical significance was designated *a priori* as a *P*-value less than or equal to 0.05.

PPE results following treatment were analyzed using a simple linear regression model (Mutapi and Roddam, 2002). The null hypothesis was that treatment with enrofloxacin had no effect on PPE over time following treatment. This hypothesis was tested using a student t test of the slope of the linear regression line to determine if the gradient was zero (Zou et al., 2003). An analysis of variance (ANOVA) *F*-test for comparing the simple linear regression model to a separate means (one-way ANOVA) model was used to assess the goodness of fit of simple linear regression. A large *P* value ( $>0.05$ ) indicated that there was no evidence of lack-of-fit to the simple linear regression model (Ramsey and Schafer, 2002).

In addition to the linear regression modeling, one-way ANOVA tests were conducted in order to determine whether there were statistical differences between days following treatment. This approach was preferred where there was evidence of lack-of-fit to the linear regression model. The Tukey-Kramer HSD (honest significant difference) test was used to compare the mean PPE, PCV and cELISA each day in order to identify statistical differences between days following enrofloxacin therapy. This test is an exact alpha-level test if the sample sizes are the same and conservative if the sample sizes are different (Hayter, 1984).

## Results

Following infection and prior to treatment, animals in the treated and control groups demonstrated a similar increase in PPE and decrease in PCV ( $P = 0.39$  and  $0.72$ , respectively). Both animals in the control group became moribund when the PCV dropped below 10% and were euthanized (normal: 24–46%). This occurred at approximately 3–6 days following peak parasitemia. At this time point, the difference in PPE between the treatment and control group was approaching statistical significance ( $P = 0.061$ ).

At the time of treatment the splenectomized calves had a mean rickettsemia ( $\pm$  SEM) of  $39.13 \pm 5.67\%$  parasitized erythrocytes (range: 28.6%-53.2%) at  $21 \pm 1.35$  days after infection. The mean PCV was  $26.45 \pm 3.02\%$  and the cELISA was  $82.66 \pm 3.879\%$  inhibition. By 6 days after treatment the mean PPE had decreased by 62% to  $14.88 \pm 4.64\%$ . The mean PCV had also decreased by 48% to  $13.80 \pm 2.17\%$ . The mean PPE remained between  $3.60 \pm 2.34\%$  and  $4.97 \pm 4.78\%$  for the next 18 days with levels fluctuating for individual animals. Recrudescence of rickettsemia was however self limiting and did not require further treatment. A progressive increase in mean PCV was observed from 6 days after treatment to the end of the study. The cELISA remained constant throughout the study. These results are summarized in Table 2 and represented graphically in Fig. 1.

Linear regression of PPE against days post-treatment provided substantial evidence that the slope of the regression line was not zero ( $P < 0.0002$ ). The lack-of-fit  $F$ -test ( $P = 0.0028$ ), however, indicated that variability between the group means for all the data could not be explained by a simple linear regression model (Ramsey and Schafer, 2002). A one-way ANOVA was conducted which provided substantial evidence ( $P < 0.0001$ ) of post-treatment PPE differences. The Tukey-Kramer HSD test indicated that only the PPE at treatment was significantly different ( $P < 0.001$ ).

Based on these findings the PPE measurements taken over the first 12 days were subjected to simple linear regression. This is similar to the approach used by Kuttler and Simpson (1978).

The equation of the regression line was determined to be:

$$\text{PPE} = 29.875 - 3.196 (\text{days post-first treatment})$$

A lack-of-fit  $F$ -test indicated that a regression line was appropriate to describe these data ( $P = 0.17$ ). Linear regression provided substantial evidence to reject the null hypothesis that treatment with enrofloxacin had no effect on PPE over time based on the slope of the regression line ( $P < 0.0002$ ). Instead, these data supported the alternative hypothesis that enrofloxacin substantially reduced the PPE over time following treatment.

Similar simple linear regression analyses were also conducted on the PCV and cELISA results. There was evidence of lack-of-fit indicating that a one-way ANOVA test

provided a more appropriate analysis. Despite the large fluctuation in PCV observed during the first 2 weeks of the study there was no evidence of a statistically significant difference in PCV and cELISA in treated animals over the course of the study.

## Discussion and conclusions

The purpose of this study was to evaluate the efficacy of enrofloxacin (Baytril®100, Bayer HealthCare LLC) against severe (> 25% PPE) experimental *A. marginale* infections in splenectomized calves. Our study found treated animals survived severe infections whereas untreated calves became moribund and were euthanized on welfare grounds. In surviving animals there was substantial evidence that enrofloxacin administered at 12.5 mg/kg twice, 48 h apart, suppressed but did not eliminate severe *A. marginale* infections in splenectomized calves. A recrudescence of *A. marginale* parasites was observed in all animals within 30 days following treatment. Subsequent rickettsemias were less severe and self limiting. The PCV returned to pretreatment levels within 6 weeks following treatment.

The present study was constrained by the small number of animals enrolled. This arose due to limited availability and cost of study animals. In the present study the control calves became moribund and were euthanized when the PCV dropped below 10%. Failure to intervene at this point in the study when the PPE was greater than 40% could not be justified on welfare grounds. Previous studies have indicated that a severe anaplasmosis infection in splenectomized calves is fatal if left untreated (Roby, 1972; Kuttler and Simpson, 1978). Given the loss of the control animals, the simple linear regression model was used to analyze these data.

The efficacy of enrofloxacin against acute *A. marginale* infections has been studied in two previous reports. Schröder et al. (1991) examined the effect of enrofloxacin on *A. marginale* in 3 trials. Splenectomized and intact calves were treated with two injections of either 5 or 10 mg/kg enrofloxacin when the PCV decreased to 25%. Either dose rate was deemed effective based on the disappearance of parasites from blood smears and the return of PCVs to pre-treatment levels. Guglielmone et al. (1996) treated intact Holstein steers with 10mg/kg enrofloxacin for 2 consecutive days when the PPE ranged from 3–10%. A significantly lower rickettsemia ( $P < 0.01$ ) was found 72 h following treatment. In both of

these studies, animals were not evaluated for recrudescence of rickettsemia or infectivity following treatment. Furthermore the disease described in these studies was not as severe as the infection model utilized in the present study.

The utility of splenectomized calves in the study of chemotherapeutic agents against *A. marginale* has been described in the literature. Splenectomized calves have been used to emphasize the drug effect and minimize possible immune responses that might camouflage specific drug actions. Furthermore, splenectomized calves present an obvious advantage in early screening trials due to the consistent relapse pattern occurring in those instances where treatment is ineffective (Kuttler, 1972).

Roby (1972) demonstrated increased survival of splenectomized calves treated parentally with imidocarb at 2.5, 5.0 and 10.0 mg/kg. Kuttler (1986) observed a similar pattern of parasite suppression and recrudescence in splenectomized calves following administration of imidocarb dipropionate (at 5 mg/kg) and oxytetracycline hydrochloride (at 20 mg/kg) which is similar to that reported in the present study.

All calves evaluated in our study remained persistently infected with anaplasmosis after treatment. Non-splenectomized cattle that recover from acute anaplasmosis remain lifelong carriers of the disease. Persistent infection is characterized by sequential rickettsemic cycles ranging from  $10^2$ – $10^7$  that occur at approximately 5-week intervals (Eriks et al., 1989). Each cycle of persistent rickettsemia reflects emergence of antigenically variant *A. marginale* that are subsequently controlled by variant-specific primary immune responses (French et al., 1999; Brayton et al., 2003). These cycles are microscopically undetectable in the intact animals (Kieser et al., 1990). In contrast, microscopically detectable cycles occur in splenectomized animals that survive primary infection, presumably due to the absence of phagocytosis of parasitized erythrocytes in the spleen (Buening, 1973). It is noteworthy that subsequent cycles appear more frequently but are self limiting in splenectomized calves following therapy.

In the United States, the labeled dose of enrofloxacin for single dose therapy of bovine respiratory disease in cattle is 12.5 mg/kg bodyweight administered subcutaneously. The bactericidal activity of fluoroquinolone antimicrobials is dependant on the ratio of the area under the plasma drug concentration curve (AUC) to the minimum inhibitory

concentration (MIC) for the organism (Drusano et al., 2001). Enrofloxacin is metabolized to ciprofloxacin in calves, which contributes between 10% and 27% of the total concentration present in the serum (Kaartinen et al., 1997). The MIC of enrofloxacin and ciprofloxacin against *A. marginale* has not been determined. Antimicrobial susceptibility studies involving ciprofloxacin have been conducted on *Anaplasma phagocytophilum*, a closely related agent responsible for human granulocytic anaplasmosis (HGE). These studies have determined the MIC of ciprofloxacin to be between 1 µg/ml and 2 µg/ml (Klein et al., 1997; Branger et al., 2004). We therefore administered enrofloxacin twice, 48 h apart in an attempt to maximize AUC above the hypothesized MIC against *A. marginale*.

Our study examined three geographically and phylogenetically distinct isolates of *A. marginale* as described by de la Fuente et al. (2001). This closely resembles the heterologous situation that is likely to be encountered in the field application of antimicrobial therapy in a diverse population of animals. It is noteworthy that Maurin et al. (2003) demonstrated differences in the MIC of the fluoroquinolone levofloxacin against different geographic isolates of *A. phagocytophilum*. *In vitro* MICs varied from 0.06–0.5 µg/ml, which is reported to be close to the maximum levels achievable in human serum. Our very limited study did not show any evidence of a difference in susceptibility of these *A. marginale* isolates to enrofloxacin *in vivo*. However this finding requires further evaluation before a definitive conclusion can be made.

In an earlier study, Maurin et al. (2001) demonstrated a DNA gyrase-mediated natural resistance to fluoroquinolones in *Ehrlichia chaffeensis* and *Ehrlichia canis*. This corresponds to a single amino acid difference in *gyrA* which encodes the A subunit of DNA gyrase in *A. phagocytophilum*. Concern has been expressed that acquired fluoroquinolone resistance in *A. phagocytophilum* could be expected following widespread use. The recent publication of the complete genome sequencing of *A. marginale* (Brayton et al., 2005) may allow DNA sequence alignment of the quinolone resistance-determining region (QRDR) of *gyrA* which encodes the A subunit of DNA gyrase. This would be helpful to assess potential differences in quinolone susceptibility between *A. marginale* and *A. phagocytophilum* and also the potential for quinolone resistance.

The results of the present study indicate that enrofloxacin administered at 12.5 mg/kg twice, 48 h apart ameliorates severe *A. marginale* infections in splenectomized calves. Untreated control animals became moribund and were euthanized. Recovering treated animals remained persistently infected after treatment with this regimen. Further studies are warranted to investigate whether a dose regimen of enrofloxacin can be identified to eliminate *A. marginale* infection from carrier cattle.

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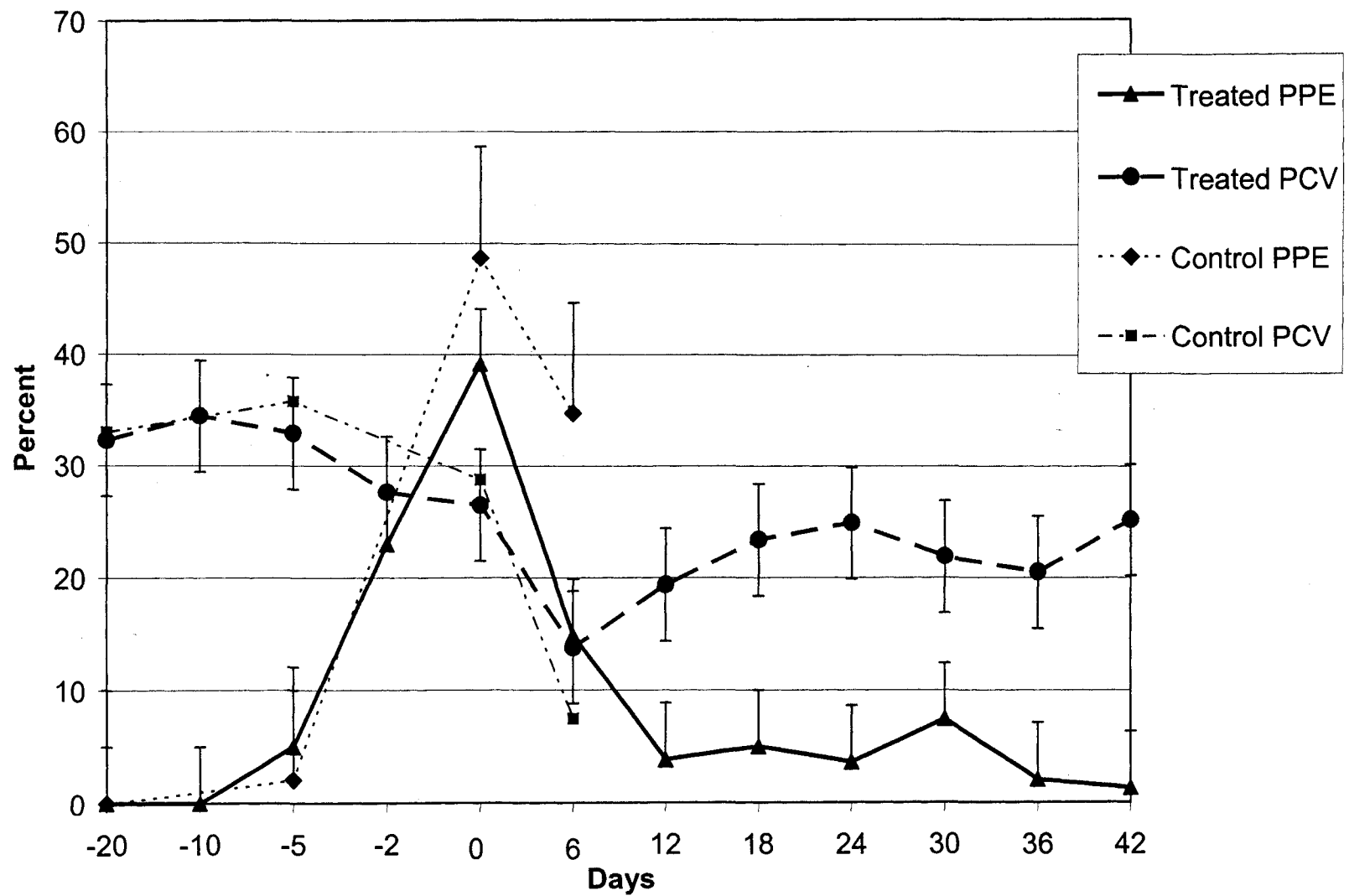
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**Figure 1: Mean percent parasitized erythrocytes (PPE) and packed cell volume (PCV) following treatment on Day 0 with 2 subcutaneous injections of enrofloxacin (Baytril, Bayer Animal Health) at 12.5 mg/kg bodyweight Q48H**

**Table 1: Individual animal details**

Calf ID	Isolate	Infection to Peak PPE	Peak PPE <sup>a</sup>	Weight	Dose of Enrofloxacin (mg)	Dose Administered (ml)
15	Oklahoma	39 days	55.50%	Control	Control	Control
63	St Maries	19 days	43.30%	169 kg	2112 mg	21ml
65	Oklahoma	15 days	41.90%	Control	Control	Control
67	Virginia	20 days	28.60%	169kg	2112 mg	21ml
70	Oklahoma	20 days	31.40%	147kg	1838 mg	18ml
71	St Maries	25 days	53.20%	68kg	850 mg	9ml

<sup>a</sup>PPE: Percent Parasitized Erythrocytes

**Table 2: Summary of the mean percent parasitized erythrocytes (PPE) and packed cell volume (PCV) following administration of enrofloxacin (Baytril, Bayer) twice at 12.5mg/kg bodyweight SQ, Q48H**

Days Post Treatment	0	6	12	18	24	30	36	42
Mean PPE	39.13**	14.88**	3.83	4.98	3.60	7.40	2.07	1.30
SEM <sup>a</sup>	5.67	4.64	2.94	4.78	2.34	6.04	1.37	0.55
Lower 95% CI <sup>b</sup>	21.08	0.09	-5.53	-10.23	-3.86	-11.81	-3.83	-1.07
Upper 95% CI	57.17	29.66	13.18	20.18	11.06	26.61	7.97	3.67
Mean PCV	26.45	13.80	19.35	23.38	24.88	21.88	20.50	25.17
SEM	3.02	2.17	3.12	3.14	1.20	2.16	4.24	0.67
Lower 95% CI	16.85	6.89	9.43	13.39	21.07	14.99	7.01	22.30
Upper 95% CI	36.05	20.71	29.27	33.36	28.68	28.76	33.99	28.04

<sup>a</sup>SEM: Standard Error of the Mean

<sup>b</sup>CI: Confidence Interval

\*\*Statistically Significant Difference ( $p < 0.01$ )

## **CHAPTER 6. A STUDY TO COMPARE THE EFFICACY OF ENROFLOXACIN, IMIDOCARB AND OXYTETRACYCLINE AGAINST PERSISTENT *ANAPLASMA MARGINALE* INFECTIONS**

A paper submitted for publication in Veterinary Parasitology

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### **Abstract**

*Anaplasma marginale* is the most prevalent tick-borne pathogen of cattle worldwide. Cattle that recover from acute anaplasmosis become carriers in which low or microscopically undetectable rickettsemia persists. There are currently no antimicrobials approved for elimination of these carrier infections. This study compared the efficacy of enrofloxacin, imidocarb and oxytetracycline against persistent *Anaplasma marginale* infections. Twelve Holstein calves, aged 4–12 months, were experimentally infected with an Oklahoma (OK), Virginia (VGN) or St Maries (StM) isolate of *A. marginale* as part of a previous experiment. Following infection, calves demonstrated a brief parasitemia and seroconverted as determined by competitive ELISA test (>30% inhibition). Approximately 66 days after infection, calves were blocked by bodyweight and randomly assigned to one of three treatment groups. Treatment A consisted of enrofloxacin (ENRO) (Baytril®100, Bayer Animal Health) administered at 5 mg/kg bodyweight, intravenously (IV), q 24 h for 5 days; Treatment B consisted of imidocarb dipropionate (IMD) (Imizol Injectable Solution®, Schering Plough Animal Health) administered at 5 mg/kg bodyweight, by intramuscular (IM) injection twice, 7 days apart; Treatment C consisted of oxytetracycline (OTC) (Liquamycin LA-200®, Pfizer Animal Health) administered at 22 mg/kg, intravenously (IV), q 24 h for 5 days (a treatment regimen that corresponds with current Office International des Epizooties (OIE) recommendations for treating persistent infections prior to export). Approximately 7–18 days after treatment calves were splenectomized and monitored for evidence of parasitemia, anemia and changes in serological status. Infectivity of blood from negative calves was tested after 7 weeks by inoculation into splenectomized calves. Eighty days after

first treatment, parasite negative animals were further tested by direct and nested PCR. A calf infected with the OK isolate treated with IMD and a calf infected with the VGN isolate treated with OTC failed to develop parasitemia following splenectomy. Both calves became seronegative at 43 days after treatment. Subinoculation of blood pooled from these calves failed to infect a splenectomized calf. The OTC treated calf and the subinoculated splenectomized calf were negative on PCR and nPCR, but the IMD treated calf was positive. These data indicate that two of the regimens described were efficacious against persistent *A. marginale* infections in two animals infected with an OK and VGN isolate.

## Introduction

Anaplasmosis, caused by the rickettsial hemoparasite, *A. marginale*, is the most prevalent tick-transmitted disease of cattle worldwide (Uilenberg, 1995; Dumler et al., 2001; Kocan et al., 2003). The existence of persistent *A. marginale* infections restricts the export of animals to areas where the disease is not regarded as endemic. Kocan et al. (2003) estimated the cost of anaplasmosis in the USA to be over \$300 million per year.

Prior to the development of imidocarb dipropionate and the tetracycline antimicrobials, a variety of chemotherapeutic agents, including arsenicals, antimalarials, antimony derivatives and dyes, were used to treat acute anaplasmosis. These compounds had little if any chemotherapeutic effect (Potgieter and Stoltz, 1994). Chlortetracycline and oxytetracycline are the only compounds available for the control of acute anaplasmosis in the United States. *A. marginale* infections are not sterilized at the usual recommended therapeutic doses of the tetracycline drugs (Kuttler and Simpson, 1978; Stewart et al., 1979). There are currently no antimicrobials labeled for the elimination of persistent infections in carrier animals.

Tetracyclines bind to ribosomes and mRNA, although inhibition of protein synthesis is mediated principally through reversible binding with the 30S ribosomal subunit (Scholar and Pratt, 2000). Previous studies in which successful clearance of persistent *A. marginale* infections was reported administered oxytetracycline intravenously to cattle at 11–22 mg/kg for 5–12 days (Magonigle et al., 1975; Roby et al., 1978). Intramuscular oxytetracycline administered at 20 mg/kg on 2, 3 or 4 occasions at intervals ranging from 3–7 days was also



reported to be effective at eliminating carrier infections (Roby et al., 1978; Kuttler et al., 1980; Magonigle and Newby, 1982; Kuttler, 1983; Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem, 1988). A recent study conducted by our research group demonstrated that the current recommended OIE treatment protocol of 5 daily injections of oxytetracycline administered intravenously at 22 mg/kg did not eliminate persistent Oklahoma isolate infections (Coetzee et al., 2005a).

Enrofloxacin is a fluoroquinolone antimicrobial that inhibits bacterial DNA-gyrase (Topoisomerase II) and Topoisomerase IV (Blondeau, 2004). This activity prevents DNA supercoiling and decatenation of original chromosomes and replicates. Two published reports indicate that enrofloxacin (Baytril<sup>®</sup>, Bayer Animal Health) is effective against acute *A. marginale* infections at dose rates of 5 to 10 mg/kg (Schröder et al., 1991; Guglielmone et al., 1996). However, the dynamics of the infection following treatment and the potential for elimination of the carrier state was not assessed.

Imidocarb is a carbanilide derivative with antiprotozoal activity. The mode of action of imidocarb is uncertain though two mechanisms have been proposed: interference with the production and/or utilization of polyamines, or prevention of entry of inositol into the erythrocyte containing the parasite (EMEA, 2001). Roby and Mazzola (1972) found that two injections of imidocarb, administered at 5 mg/kg 14 days apart, eliminated *A. marginale* from carrier animals.

The purpose of this study was to compare the efficacy of enrofloxacin administered at 5 mg/kg IV q 24 h for 5 days; imidocarb dipropionate at 5 mg/kg administered IM twice, 7 days apart and oxytetracycline administered at 22 mg/kg q 24 h for 5 days, against experimental persistent *A. marginale* infections. Multiple *A. marginale* isolates obtained from Oklahoma, Virginia and Idaho (St. Maries) were used to replicate field cases of anaplasmosis. Our study also allowed us to assess the effect of chemosterilisation on seropositivity in splenectomized calves as determined by a newly developed commercial competitive ELISA test.

## **Materials and methods**

This protocol was approved by the Committee on Animal Care (COAC) at Iowa State University.

### **Experimental cattle**

Twelve Holstein calves were obtained from the Iowa State University Dairy Breeding Research facility at Ankeny, Iowa and from a dairy herd in northwest Iowa. Both herds had no recorded cases of anaplasmosis. The individual animal details are summarized in Table 1. Calves were aged approximately 4–12 months and weighed between 59 and 206 kg at the time of treatment. Animals were confirmed free of *A. marginale* antibodies by competitive enzyme linked immunoabsorbent assay (cELISA) (Anaplasma Antibody Test Kit, VMRD Inc., Pullman, WA) (Torioni De Echaide et al., 1998).

### **Housing and husbandry**

Calves were housed in a Biolevel 2 livestock infectious disease isolation facility at Iowa State University. These were housed in pairs and fed approximately 3 lbs. of a corn-based ration twice daily. The ration was manufactured without the addition of any oxytetracycline antibiotics. Animals also received a small amount of grass hay twice a day and water *ad libitum* for the duration of the study.

### **Experimental infection with anaplasmosis**

Calves on this study were infected as part of a study to investigate the infectivity of whole blood cultures. Parasitized erythrocytes, frozen in liquid nitrogen, were obtained from splenectomized calves infected with an Oklahoma, Virginia or West Coast (St Maries) isolates. Cultures were incubated for 7 days at 37 °C, 5% CO<sub>2</sub> in 96 well plates containing RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 25 mM HEPES buffer; 200 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The contents of 20 wells (4,000 µl) from each selected sample were pelleted by centrifugation at 450 x g for 5 min. 400 µl packed parasitized erythrocytes were resuspended in 1.5 ml RPMI 1640 and inoculated by intravenously jugular injection using a 16G, 1 in. needle. Seven calves were

infected with a Virginia isolate of *A. marginale* and 4 calves were infected with an Oklahoma isolate. The remaining calf was infected with a West Coast (St Maries) isolate.

### **Post-infection monitoring**

Following infection, calves were monitored daily for the clinical signs of anaplasmosis, including anorexia, depression and listlessness. Blood samples were collected at least once a week post-infection for determination of PPE, PCV and cELISA serology. Persons conducting these analyses were blinded to treatment group. Blood samples were collected by jugular venipuncture using 18 G, 1 in. needles (Air-tite Products Co., Inc., VA). For serum collection, 10 ml Monoject® No Additive Sterile glass tubes (Sherwood Medical, St Louis, MO) were used. For whole blood (PCV and PPE), 7 ml K<sub>3</sub>EDTA glass tubes (Beckton Dickenson Vacutainer Systems, NJ) were used. Blood in EDTA was refrigerated prior to PCV testing or packaged in insulated material for overnight delivery by courier to Oklahoma State University for determination of the PPE.

Blood smears for PPE determination were stained using a 30-s, three-step staining technique (Hema 3® Staining System, Fisher Scientific) comparable to the Wright-Giemsa method. Two slides were prepared for each blood sample and examined for the presence of *A. marginale* at 100x magnification using a grid. A total of 500 cells were counted within the four squares of the grid and the number of infected cells was recorded. The PPE was the number of infected cells divided by the total number of cells counted, multiplied by 100.

Serologic testing by cELISA was conducted by the Iowa State University Veterinary Diagnostic Laboratory. Technicians conducting the test were blinded to treatment group. The test was conducted in accordance with the method described in the OIE Manual of Standards for diagnostic tests and vaccines and the manufacturer recommendations (OIE, 2000; VMRD, 2003). Results are given as percentage inhibition. PCVs were determined by partially filling heparinized capillary tubes (Chase Scientific Glass Inc., Rockwood, TN) with blood which were centrifuged for 3 min using an Adams Micro-Hematocrit centrifuge (Model CT 2900, Clay Adams Inc., New York).

Infected calves were classified as carriers based on the presence of antibodies on the cELISA test (> 30% inhibition) and after rickettsemias had fallen below 1%. This occurred at least 66 days after infection.

### **Group assignment and randomization procedures**

Carrier steers were blocked by bodyweight prior to being randomly assigned to treatment groups. Designation to treatment within a weight block occurred by assigning random numbers (Microsoft Excel, Microsoft Corporation, Redmond, WA).

### **Treatment**

Antimicrobial formulations were administered at the dose rates and dosing frequency described below. Treatment A consisted of a 100 mg/ml solution of enrofloxacin (Baytril® 100, Bayer Animal Health, Shawnee Mission, KS) administered at 5 mg/kg bodyweight, intravenously (IV), once daily (q 24 h) for 5 days. Federal (USA) law prohibits the extra-label use of enrofloxacin in food producing animals. Accordingly, experimental animals were kept in an isolation facility after treatment and were euthanized and incinerated at the end of the study.

Treatment B consisted of a 120 mg/ml solution of imidocarb dipropionate (Imizol Injectable Solution®, Schering Plough Animal Health, Union, NJ) administered by intramuscular (IM) injection at a dose rate of 5 mg/kg bodyweight, twice, 7 days apart. Treatment C consisted of oxytetracycline (OTC) (Liquamycin LA-200®, Pfizer Animal Health, Exton, PA) administered at 22 mg/kg IV, q 24 h for 5 days. This treatment regimen corresponds with current Office International des Epizooties (OIE) recommendations for treating persistent infections prior to export.

Enrofloxacin and oxytetracycline were administered to Group A and C by slow IV injection into the left or right jugular vein. A 14 gauge x 5.25 in. indwelling catheter (BD Angiocath, Beckton Dickinson, Sandy, UT) was used for each administration. The nominal content of active ingredient for each formulation and the bodyweights obtained 24 h prior to administration were used to calculate the theoretical dosage for each animal. For dosing, a 10 ml or 20 ml hypodermic syringe was used and the required dose was rounded to the nearest whole milliliter.

Imidocarb dipropionate was administered by deep intramuscular injection using an 18 gauge x 1.5 in. needle in the left and right neck, which is one of the recommended routes of administration. The nominal imidocarb content for the formulation and the animal bodyweights obtained 24 h prior to administration were used to calculate the theoretical dose for each animal. For dosing, a 10 ml hypodermic syringe was used and the required dose was rounded to the nearest whole milliliter.

### **Post-treatment splenectomy and monitoring**

At 15, 16, 22 and 24 days after first treatment, one animal from each treatment group was randomly selected and splenectomized on each day using the technique described by Thompson et al. (1992). Anaplasmosis carrier animals develop clinical anaplasmosis in approximately 14 days after splenectomy (Foote et al., 1951). This phenomenon has been used to evaluate the carrier status of calves and the success of chemosterilization regimens by splenectomizing animals after treatment (Roby and Mazzola, 1972; Stewart, 1979). Blood samples were collected at surgery and again on day 28, 43 and 49 post-treatment for PPE, PCV and cELISA serology. At 7 weeks after first treatment, all animals that had demonstrated a parasitemia were designated treatment failures and were removed from the study.

### **Determination of post-treatment infectivity**

At 7 weeks post-treatment, 5 ml of blood was collected in heparinized vacutainers from each calf that failed to develop a parasitemia following splenectomy. Blood from 2 calves was pooled and injected into a seronegative splenectomized calf. Blood was also collected from a parasitemic calf designated a treatment failure and inoculated into a splenectomized calf, which served as an infected control. Following inoculation, calves were monitored on day 56, 63, 70, 76 and 80 for the presence or absence of *A. marginale* infection as described previously.

### **Polymerase chain reaction (PCR) and nested PCR determination**

Blood samples from carrier and splenectomized animals failing to demonstrate a parasitemia at 80 days after first treatment were submitted for direct PCR and semi-nested

PCR. Testing was conducted at Washington Animal Disease Diagnostic Laboratory.

Technicians conducting the test were blinded to treatment group. Briefly, genomic DNA was isolated from 300 µl of blood using a DNA isolation kit (Purogene, Gentra Systems, Inc.) following the manufacturer's instructions. DNA of each sample was resuspended in 100 µl of hydration solution. Primers were designed from the published sequence of *msp5* from *A. marginale* Florida as described by Torioni De Eschaide et. al. (1998) and were as follows (5'-3' sequence and location): external forward, 5'-GCA TAG CCT CCG CGT CTT TC-3' (*msp5* positions 254–273); external reverse, 5'-TCC TCG CCT TGG CCC TCA GA-3' (*msp5* positions 710–692); internal forward, 5'-TAC ACG TGC CCT ACC GAG TTA-3' (*msp5* positions 367–387).

Two PCR rounds in a final volume of 50 µl consisting of 5 µl of 10x PCR buffer [200 mM Tris-HCl (pH 8.4), 0.1mM KCl ], 1.5 µl of 50mM MgCl<sub>2</sub>, 1 µl dNTPS 10mM, 1 µl of 20 pmol of each external primers, 1 µl of Taq DNA polymerase [GIBCO, 5 U per µl ], 34.5 µl of H<sub>2</sub>O and finally 5 µl of the sample DNA in case of direct PCR or 5 µl of the amplicon from the direct PCR in case of the semi-nested PCR were carried out in a Perkin Elmer GeneAmp 9600 thermocycler. Cycling conditions were preheating at 95 °C for 3 min and 35 cycles of 94 °C for 15s, 65 °C for 58 s, and 72 °C for 70 s with final extension at 72 °C for 10 min for each round. nPCR was carried out only on samples that were negative for direct PCR. PCR and nPCR products were visualized in a 2% agarose gel following electrophoresis and staining with ethidium bromide.

### Statistics

Data were entered into a spreadsheet program (Excel 2003, Microsoft Corporation, Redmond, WA) for subsequent calculation and manipulation. The mean ± SEM (standard error of the mean) were calculated for all parameters. Hypothesis tests were conducted using JMP 5.1.2 analytical software (SAS Institute, Inc., Cary, NC). Group differences between treated animals were analyzed using a student t test. Statistical significance was designated *a priori* as a *P*-value less than or equal to 0.05.

The analysis of variance approach to repeated measures data was used to analyze differences between cELISA, PCV and PPE results (Everitt, 1995). The Wilk's lambda test

was selected to evaluate within group interactions and evidence of time x group interactions. This test is a likelihood ratio statistic for testing that a multivariate contrast is zero, assuming multivariate normality and further assuming equality of covariance matrices across groups (Everitt and Dunn, 2001).

Where the Wilk's lambda test indicated a statistically significant interaction, differences between antimicrobials were analyzed using ANOVA and the Tukey-Kramer HSD (honest significant difference) method for multiple comparisons. This test is an exact alpha-level test if the sample sizes are the same and conservative if the sample sizes are different (Hayter, 1984).

## Results

### Infection phase of the study

The PPE results from the infection phase of the study are summarized in Fig. 1. cELISA and PCV results from both the infection and treatment phase are summarized in Figs. 2–3. Treatment group assignments were designated after randomization on day 66 post-infection.

Prior to inoculation, all calves were seronegative for anaplasmosis (<30% inhibition). The mean PCV ranged from  $31.08 \pm 2.36\%$  to  $35.75 \pm 4.05\%$  in the groups later assigned to treatment with imidocarb and enrofloxacin respectively. Thirty-two days after infection, all calves had seroconverted with a mean cELISA ( $\pm$  SEM) ranging from  $76.66 \pm 8.36\%$  inhibition (IMD group) to  $85.37\%$  inhibition (ENRO group). By 38 days post-infection, all calves demonstrated evidence of *A. marginale* infection based on a mean PPE ( $\pm$  SEM) ranging from  $1.37 \pm 0.67\%$  (IMD group) to  $2.05 \pm 2.2\%$  (ENRO group). Peak PPE was associated with a trough in PCV ranging from  $24.75 \pm 2.18\%$  (ENRO Group) to  $26.88 \pm 1.89\%$  (OTC Group).

Prior to treatment, 66 days post-infection, the mean PPE ranged from  $0.13 \pm 0.13\%$  (ENRO group) to  $0.25 \pm 0.17\%$  (OTC group). All animals were seropositive with the mean cELISA ranging from  $76.95 \pm 5.78\%$  (IMD group) to  $86.57 \pm 1.55\%$  (ENRO group). Mean packed cell volume (PCV) had returned to pre-infection levels and ranged from  $32.38 \pm 2.30\%$  (IMD group) to  $38.00 \pm 2.79\%$  (ENRO group).

Following assignment to treatment groups, these data were retrospectively analyzed and no statistical difference between groups was evident. The Wilks' lambda *P*-value was 0.41 for PPE, 0.63 for cELISA and 0.94 for PCV indicating that there was no group x time interaction.

#### **Post-treatment phase in carrier calves**

All treatments were well tolerated with the exception of imidocarb dipropionate, which caused mild discomfort immediately following injection. Transient cholinergic systemic reactions consisting of excessive lacrimation, serous nasal discharge, urination and increased frequency of defecation were also observed for approximately 20 min after administration. Two of the calves also demonstrated mild hind limb ataxia and apparent loss of hind limb proprioception. These signs were less severe following the second injection.

#### **Percent parasitized erythrocytes (PPE)**

At 36 days after first treatment, all four calves treated with enrofloxacin demonstrated a PPE ranging from 1–34% with a mean ( $\pm$  SEM) of  $15.85 \pm 7.55\%$  (Table 1). This occurred between 12 and 21 days following splenectomy. At this time point, one calf infected with the OK isolate (#7) and treated with oxytetracycline developed a PPE of 17.8% (12 days following splenectomy). One week later, 19 days after splenectomy, a calf infected with the VGN isolate (#14) and treated with imidocarb developed a PPE of 4%. At 7 weeks after first treatment these six calves were designated treatment failures and euthanized.

At the end of the study only two of the remaining animals had demonstrated a parasitemia. One calf (#16) infected with the VGN isolate in the OTC group developed a PPE of 3.8% and the other (#61) infected with the StM isolate in the IMD group developed a PPE of approximately 50%. Two of the four calves that failed to demonstrate a parasitemia following treatment and splenectomy were infected with the VGN isolate and were in the OTC treated group (# 2 and #12). The remaining two parasite-negative calves were infected with the OK and VGN isolate, respectively, and were treated with imidocarb (#10 and #72).



### **Competitive ELISA (cELISA)**

At 7 days after first treatment the mean cELISA ranged from  $76.47 \pm 8.57\%$  to  $90.31 \pm 0.85\%$  inhibition in the groups treated with imidocarb and enrofloxacin, respectively (Fig. 2). By 28 days after treatment the cELISA for all calves remained above 30% inhibition with the mean ranging from  $81.92 \pm 5.02\%$  inhibition (ENRO group) to  $86.13 \pm 2.50\%$  inhibition (OTC group). Between 28 and 43 days after first treatment the cELISA for calf #10, infected with the Oklahoma isolate and treated with IMD at 5 mg/kg IM twice, 7 days apart, decreased from 79.77% to 32.18% inhibition. From this point to the end of the study the cELISA for this animal ranged from 4.15–29.50% inhibition.

Similarly, over the same period of time, the cELISA for calf #2, infected with the Virginia isolate and treated with five daily IV injections of OTC at 22 mg/kg, decreased from 89.07% to 30.42% inhibition. From this point to the end of the study the cELISA for this animal ranged from 10.74–19.66% inhibition. The cELISA for the remaining animals remained above 30% inhibition for the duration of the study. Accordingly, there were no statistically significant differences between treatment groups.

### **Packed cell volume (PCV)**

At the time of splenectomy, 15–24 days after first treatment, the mean PCV ranged from  $31.5 \pm 2.02\%$  (IMD group) to  $35.75 \pm 1.25\%$  (ENRO group). At 43 days after treatment the mean PCV for all calves ranged from  $20.40 \pm 2.31\%$  (ENRO group) to  $28.95 \pm 1.41\%$  (IMD group). At 7 weeks after treatment, all animals that demonstrated a parasitemia and decrease in PCV were designated treatment failures and withdrawn from the study. The remaining animals all demonstrated fluctuations in PCV, which were not always associated with parasitemia. The most significant of these occurred in calf #2, which demonstrated a PCV of 17.5% but no parasites were evident on blood smear. At the end of the study the mean PCV of the two surviving animals was 27% and 31% in the IMD- and OTC-treated groups, respectively.

### **Post-treatment subinoculation of blood into splenectomized calves**

The results following subinoculation of carrier blood into splenectomized calves 7 weeks after carrier treatment are summarized in Table 2. Prior to inoculation, splenectomized

calves were seronegative for anaplasmosis (<30% inhibition). The mean PCV ranged from 26–34%. Fourteen days after subinoculation, calves exposed to infected blood and pooled blood from two calves treated with IMD had seroconverted (> 30% inhibition). By 20 days after subinoculation only the calf subinoculated with blood from calf #2 and #10 was seronegative. At the end of the study, 30 days after inoculation, this calf (#1) remained seronegative and failed to demonstrate a parasitemia or significant decrease in PCV.

### **Post-treatment PCR and nPCR testing**

PCR was conducted on all animals that failed to demonstrate a parasitemia at the end of the study (80 days after treatment). This included four treated calves and one subinoculated splenectomized calf. Of the infected animals, calf #2 infected with the VGN isolate and treated with OTC was negative by direct and nested PCR. Splenectomized calf #1, which had received a pooled blood sample from calf #2 and calf #10, was also PCR and nPCR negative. All the remaining treated calves were positive by direct PCR.

### **Discussion and conclusions**

Our study found that apparent clearance of persistent infections occurred in two animals treated with IMD and OTC, respectively. This conclusion was supported by the absence of parasitemia following splenectomy and a decrease in cELISA to seronegative levels (<30% inhibition) at 44 days post-treatment. Fluctuations in PCV did occur in both animals, most notably in calf #2, however these appeared to be idiopathic. It is noteworthy that only the OTC treated calf (#2) was negative on direct and nested PCR at 80 days post-treatment. However, a pooled blood sample subinoculated into a splenectomized calf at 7 weeks post-treatment did not result in parasitemia or seroconversion at 30 days post-inoculation. This calf was also negative on direct and nested PCR. Both chemosterilized calves were splenectomized 22 days after treatment. The significance or possible clinical relevance of this is unknown.

Two published reports indicate that enrofloxacin (Baytril<sup>®</sup>, Bayer Animal Health) is effective against acute *A. marginale* infections *in vivo* at dose rates of 5 to 10 mg/kg

(Schröder et al., 1991; Guglielmone et al., 1996). In this study, enrofloxacin failed to clear persistent infections in calves treated with 5 mg/kg once daily for 5 days.

Roby and Mazzola (1972) found that two injections of imidocarb, administered at 5 mg/kg 14 days apart, eliminated *A. marginale* from carrier animals. Our results indicate that shortening the interval between treatments to 7 days cleared persistent infections in 1/4 of the animals treated. Magonigle et al. (1975) evaluated the effect of five daily treatments with oxytetracycline hydrochloride at a dose of 22mg/kg IV on the carrier status of bovine anaplasmosis in 11, 2- to 3-year-old serologically-positive cattle. Our study found that this regimen was only effective in one treated animal.

There may be several reasons why the treatment regimens evaluated in this study failed to eliminate persistent *A. marginale* infections. Successful antimicrobial therapy depends on (1) achieving adequate drug concentrations at the site of infection, (2) ensuring that drug concentration is maintained for a sufficient duration to be effective, (3) pharmacokinetic parameters of the drug, (4) the susceptibility of the organism to the antimicrobial and (5) the local environment (Bidgood and Papich, 2003). Based on the pharmacokinetics and pharmacodynamics of these antimicrobials it may be hypothesized that plasma drug concentrations achieved with the regimens tested in the present study were not high enough or maintained above the minimum inhibitory concentration (MIC) for long enough in all animals to eliminate persistent infections.

Susceptibility tests facilitate the determination of the MIC, which is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (NCCLS, 2002). Our research group developed a system using whole blood culture and flow cytometric analysis (FACS) to evaluate the effect of enrofloxacin, imidocarb and oxytetracycline against an Oklahoma and Virginia isolate of *A. marginale* (Coetzee et al., 2005b). The percent reduction in HE positive cells over a 7 day period was used to select cultures to inoculate into calves in order to determine the infectivity of the culture. Based on these data we concluded that the MIC of enrofloxacin against the Oklahoma isolate of *A. marginale* was greater than 0.25 µg/ml but  $\leq 4.0$  µg/ml. However, the MIC of enrofloxacin against the Virginia isolate was greater than 4.0 µg/ml. Furthermore, we found that the MIC of imidocarb against both isolates was between 0.25 µg/ml and 1.0

µg/ml. For oxytetracycline the MIC required to be effective against anaplasmosis was greater than 16.0 µg/ml.

The bactericidal activity of fluoroquinolone antimicrobials is dependant on the ratio of the area under the plasma drug concentration curve (AUC) to the minimum inhibitory concentration (MIC). The optimal ratio in bacteria has been proposed as  $\geq 125$  (Drusano et al., 2001). Kaartinen et al. (1997) reported an AUC for enrofloxacin of 13.94 mg·h/L in 1-day-old calves and 6.73 mg·h/L in 1-week-old calves following IV administration of enrofloxacin at 2.5 mg/kg. If the AUC/MIC ratio described in bacteria applies to *A. marginale*, this would suggest that a prohibitive increase in drug dose would be required in order to achieve rickettsiicidal plasma concentrations *in vivo*. However, it should be noted that enrofloxacin is de-ethylated to ciprofloxacin in calves which contributes between 10% and 27% of the total concentration in serum (Kaartinen et al., 1997). Ciprofloxacin may also have some activity against *A. marginale* as the MIC against *Anaplasma phagocytophilum* is reported to be between 1 and 2 µg/ml (Klein et al., 1997; Branger et al., 2004). Further studies to evaluate the MIC of fluoroquinolones against *A. marginale* are necessary to fully assess the potential use of this compound for eliminating carrier infections.

Imidocarb dipropionate has been used for over 30 years in the treatment of bovine anaplasmosis in certain territories (McHardy, 1974). It is usually administered by subcutaneous or intramuscular injection to cattle at a dose rate of 2.1 mg/kg. Adverse effects as observed following first administration in our study have been reported at the 5 mg/kg dosage (Adams and Corrier, 1980). Pharmacokinetic data and reports on plasma concentrations of imidocarb that may be effective for therapy in cattle are deficient in the published literature. Studies in goats, dogs and horses suggest that this compound has a large volume of distribution resulting in a prolonged elimination half-time (Abdullah and Baggot, 1983; Belloli et al., 2002). These observations are confirmed by studies demonstrating a prolonged retention of the drug in edible tissues (EMEA, 2001). This has restricted the use of this compound in food producing animals in many territories.

Tetracycline antimicrobials are bacteriostatic rather than bacteriocidal and the activity of these compounds is believed to be dependant on the time that drug concentrations remain above the minimum inhibitory concentration (MIC) for the target organism. Blouin et al.,

(2002) used cultivated *A. marginale* in a cell line derived from embryos of *Ixodes scapularis* ticks to examine the effect of tetracycline on the organism. Infected cell cultures treated with medium containing 20 µg/ml tetracycline proved non-infective when inoculated into susceptible splenectomized calves. These data and the results of our flow cytometry research suggest that the MIC may be somewhere between 16 µg/ml tetracycline and 20 µg/ml tetracycline although this does not account for possible differences related to *in vitro* culture methods.

*In vivo* pharmacokinetic studies report a mean oxytetracycline plasma concentration of  $38.5 \pm 6.3$  µg/ml achieved at 1 h following IV administration to adult cows at 22mg/kg bodyweight (Bretzlaff et al., 1982). The mean elimination half-life ( $T_{1/2\beta}$ ) was 6.52 h. These data suggest that plasma drug concentrations will decrease to concentrations below an MIC of 20 µg/ml within 7–8 h after administration. Nouws et al. (1983) found that OTC exhibits age-dependent pharmacokinetics in ruminants. This may be related to decreases in volume of distribution and plasma clearance in older animals. Although there was no association between age or bodyweight and successful elimination of carrier infections in the present study, it is possible that individual pharmacokinetic variation could give rise to the inconsistent results observed.

A potential criticism of the present study is the use of three geographically and phylogenetically distinct isolates of *A. marginale* (de la Fuente, 2001). This may be compounded by the small number of animals enrolled. To counter this we would suggest that field cases of anaplasmosis are seldom composed of one isolate and that chemotherapy studies should be designed to account for this diversity. Conflicting reports regarding the success of chemosterilization protocols suggest that possible differences in susceptibility between isolates may exist. This hypothesis is supported by the recent identification of two multidrug resistance pumps in the genome of *A. marginale*; although the clinical significance of these pumps has yet to be elucidated (Brayton et al., 2005). Furthermore, Maurin et al. (2003) demonstrated that the MIC of the fluoroquinolone levofloxacin varied from 0.06–0.5 µg/ml depending on geographic location of *A. phagocytophilum*.

Our previous *in vitro* study revealed that statistically, the VGN isolate was more susceptible to 8 µg/ml oxytetracycline than the OK isolate. Our data also found statistical

evidence that the OK isolate was more susceptible to IMD at concentrations ranging from 0.5–4 µg/ml. Furthermore, the Oklahoma isolate cultures exposed to 4.0 µg/ml enrofloxacin failed to infect a healthy calf whereas the VGN isolate was infectious. In the present study, a calf infected with the OK isolate and treated with IMD and a calf infected with the VGN isolate and treated with OTC were cleared of infection. These findings, when viewed in conjunction with the *in vitro* data, may support a hypothesis of susceptibility differences between isolates. However, a larger study is required to investigate this further.

The present study suggests that direct PCR and nPCR are not reliable means of definitively determining the success of chemosterilization in calves. This finding is based on one calf that was positive on direct and nested PCR but failed to infect a subinoculated splenectomized calf. It is noteworthy that our study found that splenectomized calves will become seronegative following clearance of persistent infections with antimicrobials. Goff et al. (1990) observed that application of the antimicrobial interrupted continuity of stimulation of antibody as detected by complement fixation. A similar finding was reported by Coetzee et al. (2005a) using the cELISA test; however, results remained above 30% inhibition and returned to pre-treatment levels as the study progressed. Further research to establish the time frame to seronegativity in intact animals would allow the cELISA test to be used to evaluate the clearance of carrier infections in field cases of anaplasmosis.

The results of this study suggest that apparent clearance of persistent infections occurred in one calf treated with imidocarb dipropionate at 5 mg/kg administered IM twice, 7 days apart, and one calf treated with oxytetracycline (OTC) administered at 22 mg/kg q 24 h for 5 days. This conclusion was supported by the absence of parasitemia following splenectomy, a decrease in cELISA to seronegative levels (<30% inhibition) at 44 days post-treatment and the absence of disease following subinoculation of blood into a splenectomized calf. Further studies are warranted to investigate whether dose regimens can be identified to reliably eliminate persistent *A. marginale* infections from carrier cattle.

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**Table 1: Individual animal details**

Calf ID	Isolate	Treatment Group <sup>a</sup>	Bodyweight (kg)	Dose (ml)	PPE <sup>b</sup> (7 wk)	80 days	Subinoculation	Outcome
3	Oklahoma	A	202	10	22 <sup>c</sup>	Withdrawn	2.5 ml (Calf 9)	Died
11	Virginia	A	180	9	1 <sup>c</sup>	Withdrawn		
13	Virginia	A	163	8	34 <sup>c</sup>	Withdrawn	2.5 ml (Calf 9)	Died
17	Oklahoma	A	69	3	26 <sup>c</sup>	Withdrawn	2.5 ml (Calf 9)	Died
10	Oklahoma	B	152	6	0	PCR +ve	5ml (Calf 1)	nPCR -ve
14	Virginia	B	154	6	32 <sup>c</sup>	Withdrawn		
61	St Maries	B	198	8	0	PPE = 50%	5ml (Calf 8)	Died
72	Virginia	B	201	8	0	PCR +ve	5ml (Calf 8)	Died
2	Virginia	C	152	17	0	PCR -ve	5ml (Calf 1)	nPCR -ve
7	Oklahoma	C	175	19	18 <sup>c</sup>	Withdrawn	2.5 ml (Calf 9)	Died
12	Virginia	C	206	23	0	PCR +ve	5ml (Calf 4)	Died
16	Virginia	C	59	6	0	PPE = 3.8%	5ml (Calf 4)	Died

<sup>a</sup>Group A: 100 mg/ml solution of enrofloxacin (Baytril®100, Bayer Animal Health) administered at 5 mg/kg bodyweight, intravenously (IV), q24h for 5 days

Group B: 120 mg/ml solution of imidocarb dipropionate (Imizol Injectable Solution®, Schering Plough Animal Health) administered at 5 mg/kg bodyweight, by intramuscular (IM) injection twice, 7 days apart

Group C: 200 mg/ml solution of oxytetracycline (Liquamycin LA-200, Pfizer Animal Health) administered at 22 mg/kg, intravenously (IV), q24h for 5 days

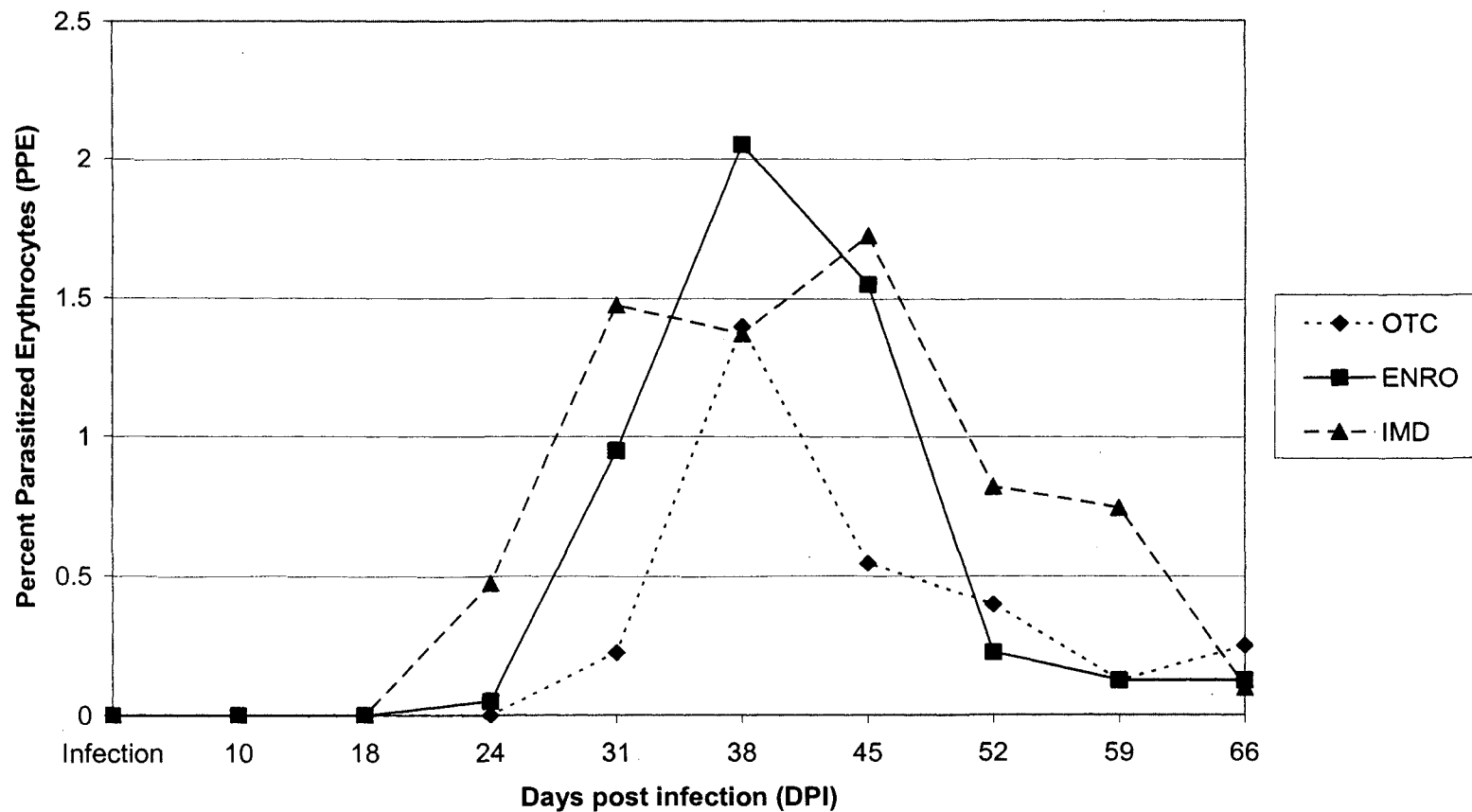
<sup>b</sup>PPE:- Percent Parasitized Erythrocytes

<sup>c</sup>Euthanised due to clinical signs of anaplasmosis (PPE, PCV, cELISA)

**Table 2. Summary of competitive ELISA, packed cell volume (PCV), percent parasitized erythrocytes (PPE) and PCR following subinoculation of carrier blood into splenectomized calves 7 weeks after carrier treatment**

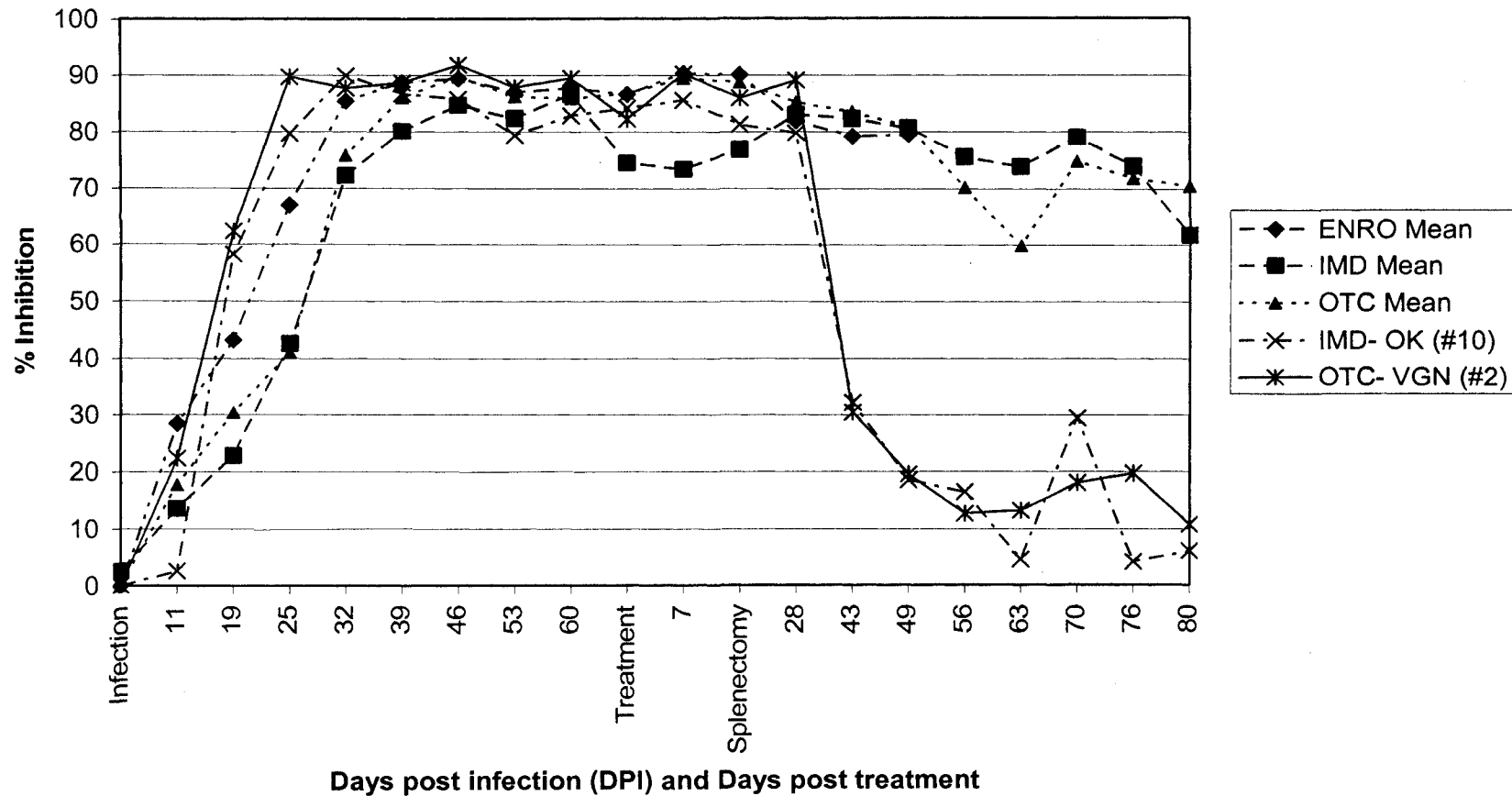
Animal	Donor	Donor Treatments <sup>a</sup>	Pre-inoculation		7 days		14 Days		20 days		End of Study		Outcome
			cELISA	PCV	cELISA	PCV	cELISA	PCV	cELISA	PCV	cELISA	PCV	
1	10 & 2	OTC & IMD	0	26	0	26	10.54	24	0	30	2.521	30	PCR/nPCR -ve
4	16 & 12	OTC	8.4	28.0	0	29	3.56	30	66.097	32	85.586	21.5	Parasite +ve
8	61 & 72	IMD	4.0	29.5	3.29	31	44.55	33	66.457	25	69.881	9.7	Parasite +ve
9	3, 7, 13, 17	Control	4.6	34.0	11.06	32	72.08	19	Died		72.08	13	Parasite +ve

<sup>a</sup>OTC: 200 mg/ml solution of oxytetracycline (Liquamycin LA-200, Pfizer Animal Health) administered at 22 mg/kg, intravenously (IV), q24h for 5 days;  
IMD: 120 mg/ml solution of imidocarb dipropionate (Imizol Injectable Solution®, Schering Plough Animal Health) administered at 5 mg/kg bodyweight,  
by intramuscular (IM) injection twice, 7 days apart;  
Control: Infected blood.



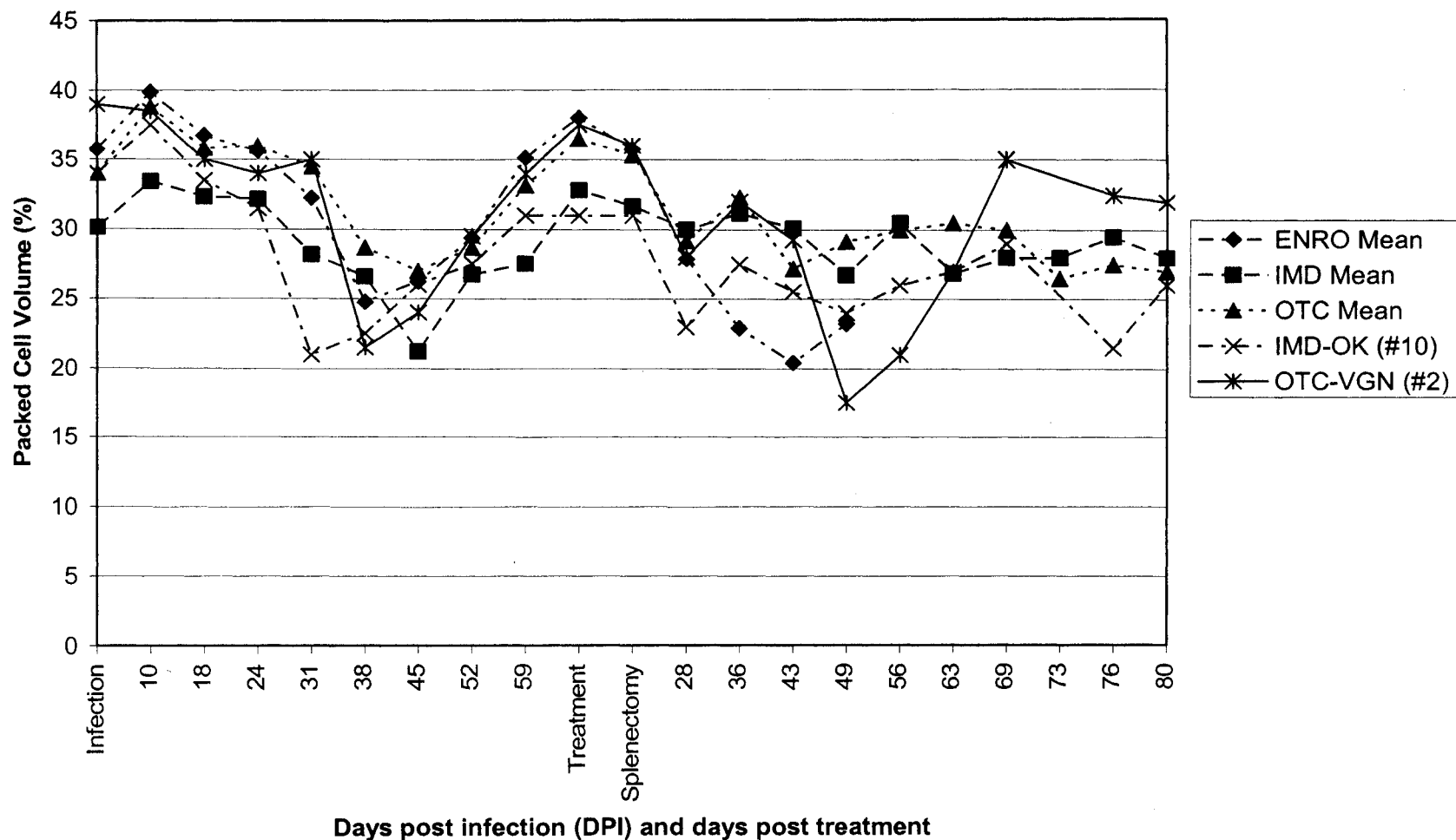
**Figure 1: Mean percent parasitized erythrocytes (PPE) following intravenous inoculation of 400 mcl cultured erythrocytes infected with *A. margini* and retrospective assignment to treatment groups 66 DPI**

ENRO—Assigned to enrofloxacin treated group; IMD—Assigned to Imidocarb treated group; OTC—Assigned to Oxytetracycline treated group



**Figure 2: Mean competitive ELISA (cELISA) results following infection with 40 0ul *A. marginale* infected erythrocytes and treatment with enrofloxacin, oxytetracycline and imidocarb 66 DPI**

ENRO—Enrofloxacin administered at 5mg/kg IV q24h for 5 days; IMD—Imidocarb dipropionate at 5mg/kg administered IM twice, 7 days apart; OTC—Oxytetracycline administered at 22mg/kg q24h for 5 days.



**Figure 3: Mean packed cell volume (PCV) following infection with 40 0ul *A. marginale* infected erythrocytes and treatment with enrofloxacin, oxytetracycline and imidocarb 66 DPI**

ENRO—Enrofloxacin administered at 5mg/kg IV q24h for 5 days; IMD—Imidocarb dipropionate at 5mg/kg administered IM twice, 7 days apart; OTC—Oxytetracycline administered at 22mg/kg q24h for 5 days.



## CHAPTER 7. GENERAL CONCLUSIONS

The results of the study presented in chapter 3 demonstrate that subinoculation of blood from all except one of the carrier steers treated with one of the 3 regimens of oxytetracycline resulted in infection and clinical anaplasmosis in splenectomized calves. The splenectomized calf in which infection could not be confirmed did however die in the early stages of the study and it may be possible that it was still in the prepatent period. Administration of a 300 mg/ml solution of oxytetracycline (Tetradure LA-300) administered at a dose of 30 mg/kg intramuscularly once or twice 5 days apart was not effective for elimination of the persistent *A. marginale* in beef cattle. Furthermore, the study demonstrated that the current recommended OIE treatment protocol of 5 injections of a 200 mg/ml solution of oxytetracycline administered at a dose of 22 mg/kg intravenously was also not effective for elimination of persistent *A. marginale* infection with an Oklahoma isolate.

These results are contradictory to findings of previous studies in which successful clearance of anaplasmosis carrier infections was achieved using intravenous oxytetracycline dosing regimens ranging from 11 to 22 mg/kg given for 5 to 12 days (Magonigle et al., 1975; Roby et al., 1978). Previous studies reported clearance of the carrier state in cattle using intramuscular oxytetracycline at 20 mg/kg following 2, 3 or 4 administrations at intervals ranging from 3–7 days (Roby et al., 1978; Kuttler, 1980; Magonigle and Newby, 1982; Kuttler, 1983; Swift and Thomas, 1983; Rogers and Dunster, 1984; Ozlem et al., 1988). Our results are however in agreement with those of Kuttler et al. (1980) and Goff et al. (1990) who report a failure of carrier clearance in naturally infected range cattle using 2 injections of long acting oxytetracycline administered at 20mg/kg by intramuscular injection.

As a result of our findings, the Canadian government removed anaplasmosis treatment requirements from feeder cattle imported from most U.S. states in March 2004 (CFIA, 2005). Although these new provisions facilitate year-round movement of feeder cattle from 39 U.S. states to Canada, there are control provisions for the movement of imported feeders between approved feedlots and for Canadian cattle to leave importing feedlots to re-enter Canada's breeding herd. Imported animals are eligible to leave the feedlot only for immediate slaughter, movement to another feedlot that is approved to import restricted feeder

cattle or to be re-exported to the U.S. There are requirements for comprehensive management programs for animals in the feedlot, enhanced insect vector control programs and periodic inspection of animals within importing feedlots.

In order to explain our findings, we hypothesized that the failure to sterilize persistent *A. marginale* infections could be attributed to one or more of the following factors: (1) drug concentrations at the site of infection were inadequate, (2) drug concentration was maintained for an insufficient duration to be effective, (3) the organism was not susceptible to the antimicrobial, (4) the pharmacokinetic parameters of the drug were inadequate and (5) local factors in the environment where the organism was located were not conducive for optimum drug activity (Bidgood and Papich, 2003).

In the second experiment we hypothesized that an *in vitro* whole blood culture system for *A. marginale* previously described by Kessler et al. (1979) could be used in conjunction with a fluorescent activated cell sorting (FACS) method described by Wyatt et al. (1991) to evaluate antimicrobials against *A. marginale*. Once established, this technique could also be used to test the hypothesis that anaplasmosis isolates from different geographic locations differ in their susceptibility to antimicrobials. Enrofloxacin inhibited *A. marginale* in a concentration dependent manner, while higher concentrations of imidocarb were less effective in reducing the number of viable organisms. Oxytetracycline was found to be the least efficacious antimicrobial in this culture system. Differences between isolates were evident at some dilutions. Cultures of erythrocytes infected with the Oklahoma isolate exposed to 4.0 µg/ml enrofloxacin and those of the Virginia and Oklahoma isolates exposed to 1.0 µg/ml imidocarb appeared to be sterilized.

These data indicate that short-term erythrocyte cultures could be used to assess novel antimicrobial agents against *A. marginale* using FACS and the vital dye, hydroethidine. Further studies are required to assess other *A. marginale* isolates for differences in antimicrobial susceptibility. Our studies were constrained by the number of animals we had available for subinoculation studies and we would suggest that additional testing should be conducted using a narrower range of dilutions. This is especially the case with Imidocarb where the MIC is judged to be between 0.25 and 1 µg/ml. Our study also demonstrated that

future *in vitro* erythrocyte culture studies may only need to be conducted over 3 days based on the results of the ROC analysis.

Based on the results of the *in vitro* trial we hypothesized that enrofloxacin could potentially be used to sterilize persistent *A. marginale* infections. Accordingly we designed the study reported in chapter 5 to test the efficacy of enrofloxacin (Baytril®100, Bayer Animal Health) against severe experimental *A. marginale* infections in splenectomized calves. The results of this study indicated that enrofloxacin administered at 12.5 mg/kg twice, 48 h apart ameliorates, but does not clear, *A. marginale* infection in splenectomized calves. Further studies are required to determine whether an efficacious dose rate, route, duration and frequency of enrofloxacin administration can be found to eliminate persistent *A. marginale* infections.

Finally we compared the efficacy of enrofloxacin, imidocarb and oxytetracycline against persistent *Anaplasma marginale* infections established experimentally using an Oklahoma (OK), Virginia (VGN) or St Maries (StM) strain. A calf infected with the OK isolate treated with imidocarb dipropionate at 5 mg/kg administered IM twice, 7 days apart, and one calf infected with the VGN isolate treated with oxytetracycline administered at 22mg/kg q 24 h for 5 days failed to develop parasitemia following splenectomy. Subinoculation of blood pooled from these calves failed to infect a splenectomized calf. The OTC treated calf and the subinoculated splenectomized calf were negative on PCR and nPCR but the IMD treated calf was positive. These data indicate that two of the regimens described were efficacious against persistent *A. marginale* infections in only two animals infected with either the OK or VGN isolate. No calves treated with 5 mg/kg intravenous enrofloxacin for 5 days were cleared.

Conflicting reports regarding the success of chemosterilization protocols suggest that possible differences in susceptibility between isolates may exist. This hypothesis is supported by the recent identification of two multidrug resistance pumps in the genome of *A. marginale*; although the clinical significance of these pumps has yet to be elucidated (Brayton et al., 2005). Future studies where these efflux pumps can be cloned would be useful to determine their significance. Once genomic sequences of other *A. marginale* isolates are elucidated, these can be compared with this first sequence obtained from a St.

Maries isolate. Future *in vivo* studies should involve groups of cattle infected with multiple isolates. The studies presented herein are limited in the number of animals utilized, which significantly restricts the extent of the inferences that can be drawn from these.

The study presented in chapter 6 suggests that direct PCR and nPCR are not reliable means of definitively determining the success of chemosterilization in calves. This finding is based on one calf that was positive on direct and nested PCR but failed to infect a subinoculated splenectomized calf.

It is noteworthy that our study found that splenectomized calves will become seronegative following clearance of persistent infections with antimicrobials. Goff et al. (1990) observed that application of an antimicrobial interrupted continuity of stimulation of antibody as detected by complement fixation. A similar finding was reported in our study presented in chapter 3 using the cELISA test; however, results remained above 30% inhibition and returned to pre-treatment levels as the study progressed. Further research to establish the timeframe to seronegativity in intact animals would allow the cELISA test to be used to evaluate the clearance of carrier infections in field cases of anaplasmosis.

Significant deficiencies exist in our understanding of many of the chemotherapeutic agents used in the treatment of bovine anaplasmosis. The tissue disposition of imidocarb dipropionate has been extensively evaluated due to violative residue potential in food animals. However, there are currently no published reports detailing the pharmacokinetics of this compound in cattle. The obligate intraerythrocytic nature of *A. marginale* lends itself very well to the conduct of pharmacokinetic/pharmacodynamic (PK/PD) modeling. Blood samples in heparin can be simultaneously evaluated for parasite activity using the FACS method described herein and plasma drug concentrations can be determined by HPLC. These data may provide very useful information regarding the relationship between the drug and the organism.

A general deficiency of previous studies examining the elimination of persistent *A. marginale* was the lack of random assignment to treatment groups. Furthermore, many of the techniques, such as serology, which was used to determine whether an animal was a carrier, have since been found to be inadequate. The advent of more sensitive molecular diagnostic techniques such as PCR may be helpful in the future. These may be particularly applicable

where field trials are conducted as these tests may be a useful indicator of carrier status when used in conjunction with the new cELISA test.

Taken together, these studies suggest that reliable clearance of persistent *A. marginale* infections could not be achieved with oxytetracycline, imidocarb dipropionate or enrofloxacin at the dose, route, frequency and duration of administration tested. We also showed that flow cytometry proved to be useful for screening antimicrobial activity of drugs against bovine erythrocytes infected with *A. marginale*. Based on the data obtained from these analyses we were able to make predictions about the minimum inhibitory concentration of antimicrobials required to be effective against anaplasmosis. We also demonstrated *in vitro* susceptibility differences between a Virginia and Oklahoma isolate, especially in the imidocarb treated cultures. We also identified enrofloxacin as a compound that could potentially be used against *A. marginale*. Further research is required to investigate why current chemosterilization protocols against *A. marginale* provide inconsistent results. This may require further elucidation of the relationship between pharmacotherapy and the immune system and the determinants of between-isolate susceptibility differences.

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